Homer, TRP Channels and Calcium: The Signalling Triad of Growth Cone Motility.

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy
University of Tasmania

July 2008

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R. Gasporini
July 2008

Acknowledgements

For Blanka, who just missed out on the party...

This thesis is more than just the physical evidence of three years of experimental work. The seed for this work was sown in a small takeaway hole-in-the-wall called Jerusalem Gardens in Ann Arbor, Michigan. It was November 2006 (or thereabouts), a warm autumn afternoon, and over a wonderful plate of felafel, I met Greg Gibson, an assistant professor in Molecular Genetics who worked at the University of Michigan. That day was to be the beginning of a personal and professional relationship that fundamentally changed my perception of who I was and what I was capable of doing.

Greg's belief in the power of genetics underpinning our perception of ourselves as organisms on this planet immediately attracted me to him, but more importantly, he showed me how we sometimes achieve profound insights into the great mysteries of our existence through the understanding of minute, simple yet powerfully relevant organisms, such as the fly. His enthusiasm was, and still is infectious. To this day, I am in awe of what he has achieved and what he will undoubtedly achieve back in Australia as a Professorial Fellow. This work would not have been possible without his scientific insights and his willingness to allow me to work closely with him. Thanks, Gibbo.

The other individual whose ongoing love, support and encouragement I acknowledge is Lisa. Her role was always going to be a difficult one to tread, not only being wife and confidant in the job of raising two gorgeous girls but also being my immediate supervisor. Somehow we got though it all with only a few arguments about experiments, cells and the lack of money to do our research. Somehow we survived on small grants while she ran the inevitable gauntlet of the research/teaching chasm.

She taught me how to write, design simple, yet relevant experiments and above all she was able to keep our professional and personal lives apart. For all you have shown me, and all of your love, thanks Lisa.

There were many other individuals without whose advice, material support and physical assistance, this work would not have been possible. In particular, my thanks go out to

James Vickers and his present and past lab members, who allowed me to invade their lab, pull apart microscopes, set up imaging rigs and never begrudge me antibodies when I ran out. Thanks James, and I look forward to working with you more closely. To my primary and associate supervisors, Adrian West and Inn Chuah, thank you for your support of my studentship and your willingness to see this project through. To all the other individuals, undergraduates, postgraduates and technical staff, Dave Lovell, Niels, Jill, Ingrid, thanks for putting up with this grumpy and gruff individual. I owe so much to so many people...

Rob Gasperini,
Hobart, Tasmania.
July 2008

Abstract

The nervous system is an elaborate network of intricate circuits linking, monitoring and controlling all functions in the body. This circuitry, established early in development, is defined by a process known as axon guidance. The precision and accuracy of this circuitry is ultimately a correlate of the navigational capabilities of specialised structures at the distal tips of extending axons, the growth cones.

Growth cones are equipped with an array of fine antennal projections, or filipodia, sensitive to a variety of attractive or repulsive signals. These guidance cues are detected and interpreted by intracellular signal transduction mechanisms that mediate cytoskeletal rearrangements within the growth cone, ultimately providing directional control of growth cone trajectories. Guidance cues may be diffusible molecules from distant target tissues or components of contacting cells, however, the complete repertoire of molecules that transduce these extracellular signals to the cytosolic cytoskeletal machinery are yet to be fully understood.

Neurons have evolved a variety of important intracellular signal transduction pathways, many of which rely on calcium as a key second messenger molecule. Many crucial pre- and post-synaptic functions in neurons are mediated by changes in intracellular calcium concentration ($[Ca^{2+}]_i$) including filipodial protrusion and neurite elongation. Indeed, spatial $[Ca^{2+}]_i$ gradients within the growth cone are crucial for the appropriate recognition and motile responses to the key guidance molecules netrin-1 and brain derived neurotrophic factor (BDNF). Cytosolic calcium is highly regulated with the key calcium buffering organelle being the endoplasmic reticulum (ER). The mechanisms regulating the transduction of extracellular guidance signals to changes in ER mediated calcium

release, however, are still to be determined. This thesis describes work focusing on the elucidation of a molecular correlate of such a mechanism.

Homer proteins are best known as facilitators of receptor clustering and signalling at the post synaptic density. Long form Homer (H1b/c) forms dimers via C-terminal coiled-coil domains, cross-linking multiple signalling partners through N-terminal, enabled-VASP homology (EVH1) domains. This molecular motif enables Homer proteins to couple cell-surface receptors such as metabotropic glutamate receptors (mGluR) and transient receptor potential cation channels (TRPC) to intracellular calcium stores via inositol triphosphate (IP_3R) and ryanodine (RyR) receptors. Homer is necessary for axon pathfinding in the amphibian visual system in vivo, in a mechanism that to date, has remained elusive. The unique binding characteristics of this synaptic molecule, the subcellular location and physiological relevance of its binding partners makes Homer a good candidate molecule to facilitate the coupling of extracellular guidance cues to changes in $[Ca^{++}]_i$

This study addresses the following questions: What is the biochemical nature of Homer function in axon guidance? Does Homer facilitate the transduction of extracellular guidance cues to the cellular machinery required to adjust motility and guidance? Is Homer required for calcium signalling in the growth cone?

The study describes the development and characterisation of a growth cone turning assay using a relevant developmental system, i.e. primary cultures of embryonic rat dorsal root ganglion sensory neurons (DRG). Combining this assay with a targeted morpholino knockdown approach, the study shows that a crucial level of H1b/c is necessary for calcium dependant motile responses to netrin-1 and BDNF, with Homer

morphant DRG showing a reversal of motile responses from attraction to repulsion (control morphants +15.8 and +18.7 degrees for netrin and BDNF respectively while for Homer morphants -19.7 and -18.6 degrees for netrin and BDNF). Furthermore, pharmacological experiments suggest that Homer functions through the activational state of a CaMKII/calcineurin molecular switch. Such a molecular switch has recently been found to be crucial in other axon guidance model systems and is sensitive to the depth of growth cone calcium gradients, lending support to a role for Homer in the "set-point hypothesis" of growth cone motility.

On the basis of these experiments, it was hypothesised that perturbation of growth cone calcium dynamics would be a feature of Homer knockdown. Indeed, single wavelength calcium imaging experiments showed that Homer morphant DRGs exhibited altered calcium responses to BDNF microgradients and a higher frequency of TRPC-mediated calcium transients, or spike events (control morphants 0.3 events/min and Homer morphants 1.5 events/min). These results describe a crucial role for Homer in growth cone calcium homeostasis.

The relevance and importance of Homer in sensory systems is further demonstrated through an examination of the ontogeny of a putative Homer1b/c homologue in the developing zebrafish embryo. Significantly, Homer protein is prominent in the developing sensory architecture of the zebrafish larva at important developmental, behavioural and synaptogenic timepoints, supporting previous experimental data showing a crucial role for Homer in the developing amphibian visual system.

In summary, the work demonstrates that Homer acts as a facilitator of calcium signalling and thus motile events in the growth cone. These findings, therefore, may ultimately

have implications for the design and implementation of pharmacological interventions in neurological diseases and clinical conditions such as spinal cord injury.

Publications arising from this thesis

GASPERINI, R., and FOA, L., (2004) "Homer1b/c expression correlates with zebrafish olfactory system development" *Journal of Neurocytology* **33**, 671-680

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List of abbreviations

AChR	Acetylcholine receptor
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor
4-AP	4-aminopyridine
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
[Ca2+]i	Cytosolic calcium concentration
CAMKII	Calcium-calmodulin dependent kinase II
cAMP	Cyclic adenosine monophosphate
CaN	Calcineurin
C-C	Coil-coiled
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CRAC	Calcium release-activated calcium channel
CREB	Calcium response elements
CsA	Cyclosporin A
CSPG	Chondroitin sulfate proteoglycans
DAG	Diacylglycerol
DCC	Deleted in colorectal cancer
DRG	Dorsal root ganglia
DVDT	Dorsoventral diencephalic tract
EDTA	Ethylenediamine tetra acetic acid solution
EGF	Epidermal growth factor
ena	Enabled
Eph	Ephrin receptor
ER	Endoplasmic reticulum
EVH	Enabled vasodilator-stimulated phosphoprotein homology
FLIP	Fluorescence loss in photobleaching
FRET	Fluorescence Resonance Energy Transfer
GAP-43	Growth associate protein-43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GTP	Guanosine triphosphate

Homer-IR	Homer1b/c-like immunoreactivity
hpf	Hours post fertilisation
HRP	Horseradish peroxidase
HSPG	Heparan sulfate proteoglycans
IP3R	Inositol triphosphate receptor
IQGAP-1	IQ motif containing GTPase activating protein 1
LGN	Lateral geniculate nucleus
LTP	Long term potentiation
MAP2	Microtubule associated protein
MAPK	Mitogen activated protein kinase
mGluR	Metabotropic glutamate receptor
MS-22	Tricaine methanesulphonate
MT	Metallothionein
N-CAM	Neural cell adhesion molecule
NFAT	Nuclear factor of activated T-cells
NMDAR	N-methyl D-Aspartate receptor
PBS	Phosphate buffered saline
PI_3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	phospholipase-Cγ
PMA	Phorbo 12-myristate 13-acetate
PMCA	Plasma membrane calcium ATPases
PMSF	Phenyl methane sulfonyl fluoride
PSD	Post synaptic density
PVDF	Polyvinylidene difluoride
OEC	Olfactory ensheathing cell
OSN	Olfactory sensory neuron
NGF	Nerve growth factor
NT	Neurotrophin
RGC	Retinal ganglion cell
RIPA	Radioimmuno precipitation assay
ROI	Region of interest

RyR	Ryanodine receptor
SCG	superior cervical ganglion
SDS	Sodium dodecyl sulfate
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPases
SNM	Sensory neuron media
SOC	Store operated channels
TIRF	Total internal reflection fluorescence
TrK	Tyrosine kinase
TRPC	Transient receptor potentical canonical
TTX	Tetrodotoxin
TyR	Tyrosine kinase receptor
VGCC	Voltage gated calcium channel

Chapter 1

Introduction and literature review

1.1 Introduction

This thesis documents experiments aimed at understanding the molecular mechanisms required for the development of neural circuitry. As background, the reader will be taken through an overview of the literature focussing on the role of the growth cone in axon pathfinding and in neural development in general. For the purpose of completeness, parts of the literature regarding growth cone motility and calcium signalling will be expanded in the relevant experimental chapters. This review will first examine early guidance events in neural tract and scaffold formation followed by a review of the important diffusible guidance molecules in the development of early circuits. Finally, key aspects of intracellular calcium signalling and major functions of Homer in the post-synaptic density (PSD) and its interactions with calcium signalling molecules will be examined.

1.2 Axon Guidance: Historical Perspectives

A principal aim of contemporary neuroscience is to understand how neural systems are integrated into physiological processes. Understanding normal developmental processes crucial to the formation and maintenance of functional brain circuitry will ultimately allow the development of strategies to repair neural tissue damaged as a result of injury or neurodegenerative disease. Early neuroanatomists like Ramon y Cajal, using the simple tools of the day, systematically described and experimented with the vertebrate nervous system in an attempt to understand the function/structure relationships of the many neural cell types that were observed in those early preparations (Ramon y Cajal, 1909). Studying the developing nervous system provides a way of attributing functional characteristics to subsets of

cells and in doing so, underpins our understanding of the basic building blocks and topographic maps that define the central nervous system. Building on that early seminal work, advances in molecular biology, genetics, electrophysiology, *in vivo* and *in vitro* cellular imaging techniques, have provided neuroscience with insights into the fundamental mechanisms that drive the functional wiring of the nervous system

For the brain to achieve its full potential as a controlling organ, it must develop in a way that integrates its functional circuitry in a well-ordered, reproducible manner. Axons protrude from the neural somata and extend through the embryonic environment, guided by motile, sensorimotor organelles at their distal tips called growth cones (for review see Tessier-Lavigne and Goodman, 1996). This guidance mechanism allows axons to connect to their ultimate cellular targets. Recognition of the target, followed by neural activity and synaptic refinement, terminates this process forming a functional synapse. Cajal proposed a mechanism characterised by long distance chemical attraction, or "chemical neurotropism" (for reviews see Sotelo, 2002, 2003).

However, it is now understood that the complete repertoire of events leading up to target innervation utilises a variety of chemo-repulsive or attractive and contact-repulsive or attractive mechanisms acting in concert at a variety of embryonic "choice-points" along their trajectories. This view underpins the contemporary dogma of growth cone function (Lumsden and Davies, 1983, 1986; Tessier-Lavigne and Goodman, 1996; Tessier-Lavigne *et al.*, 1988). Much of the work in the field of axon guidance today is concerned with explaining the cellular basis for these observations. Multiple metabolic, cytoskeletal and genomic modifications occurring within the growth cone are

required for changes in its motility that ultimately changes axon trajectory and targeting (Tessier-Lavigne and Goodman, 1996; Tessier-Lavigne *et al.*, 1988). However, the complexity of these potential signalling modalities underpins several exciting questions. How do growth cones make any sense from all this information? What are the mechanisms that dictate whether a single guidance cue can be both attractive and repulsive? How do the myriad intracellular signalling mechanisms integrate into functions that ultimately drive growth cones and axons to their targets?

1.3 Growth cones are morphologically dynamic structures

Cajal proposed that the growth cone was the crucial structure that enabled an axon to navigate through the embryonic environment Using 3-4 day old chick embryos he noted,

> *"I have demonstrated that as every primordial axon stretches across the spinal cord, it ends in a special conical swelling, the growth cone, with a base that faces the periphery and is garnished with a large number of thin protrusions and lamellar processes that might be considered a form of rudimentary terminal arborisation. The growth cone is like an amoebic mass that act as a battering ram to spread the elements along its path, insinuating its lamellar processes between them.*
>
> (Ramon y Cajal, 1909)

The cytologic detail of his preparations enabled Cajal to observe that growth cone morphology changed with anatomical location.

"In mesoderm, growth cones associated with motor neurons often resemble a barley seed ending in a thin, very pale tip..... having crossed the grey matter ..... the growth cone becomes larger and more darkly stained because lamellar processes around the edges either disappear or become smaller .... At the ventral edge of the ependymal canal, where cones encounter obstacles requiring some time to circumvent, their leading edge becomes studded with rounded masses of cytoplasm .... the growth cones of ventral root fibres loses its ruffled appearance .... as it leaves the spinal cord,.. it becomes spindle shaped ..." (Ramon y Cajal., 1909)

Cajal inferred links with the morphology of growth cones, their location and the tasks they had to perform, suggesting they were able to sense changes in their environment or substrate (Ramon y Cajal, 1909). His early observations are supported by more recent studies, showing that growth cones are highly dynamic, with varying filopodial and lamellipodial morphologies depending on location *in vivo* and substrate composition *in vitro* (Brittis *et al.*, 1995; Burden-Gulley *et al.*, 1995). Neurons from the grasshopper limb bud show growth cone interactions mostly consisting of filopodial contacts to high-affinity substrates, whereas in areas of relatively homogeneous substrates, growth cones assume more lamellipodial morphologies, suggesting that growth cones use filopodia as the primary sensing, navigating and steering structures (O'Connor *et al.*, 1990). Perinatal growth cones extend at faster rates than embryonic growth cones, but spend less of their time pausing and retracting. Faster extending growth cones also possess more lamellipodial, rather than filopodial morphologies (Argiro *et al.*, 1984). This observation of growth cone morphological variation was also demonstrated using the avian and amphibian model

systems which have shown that growth cones assume a much more elaborate morphology when they invade an area that presents multiple choices for further progression such as the optic chiasm in *Xenopus* and nerve plexus in the chick, while having a less elaborate morphology in areas presenting fewer choice points such as retinal surface in *Xenopus* and avian muscle nerve tracts (Davenport *et al.*, 1993; Dingwell *et al.*, 2000; Holt, 1989; Kapfhammer *et al.*, 1986a; Kapfhammer and Raper, 1987a; Tosney and Landmesser, 1985).

These studies suggest that growth cones are exquisitely sensitive to changes in their environment and that their morphology is controlled by contacts with neighbouring cells or diffusible factors either in the immediate vicinity, or emanating from more distant targets. With the evolving picture of the growth cone being an autonomous navigational organ capable of guiding axons through the embryonic environment, further questions arise: what are the signals and molecules responsible for these growth cone behaviours and to what extent are these behaviours dependent on local or distant signals?

1.4 Pioneering cells, scaffolds and contact-mediated axon guidance

To examine the effects of short range or contact mediated signals on axon guidance, research has focussed on interactions of growth cones with cellular substrates. Growth cones interact with axons for directional information to facilitate the formation of tracts (Raper *et al.*, 1983; Bastiani *et al.*, 1984; Elkins *et al.*, 1990; Kuwada, 1986; Lan *et al.*, 2001; Lumsden and Davies, 1983, 1986; McIntire *et al.*, 1992; Wilson *et al.*, 1990). The early cellular events that form the initial axon pathways, however, have only recently been described. Wilson and colleagues (1991) showed that a single

axon lays down a guidance scaffold in the dorsoventral diencephalic tract (DVDT) of the zebrafish *(Danio rerio)* without any observable modification of the epithelium or changes in growth cone morphology to indicate that it was following a pre-defined path in a contact-mediated manner. These studies were significant milestones in the demonstration that early axons, or pioneers, following developmentally regulated cues, can lay down scaffolds for subsequent axon tract development. The notion of tracts and scaffolds being key structural platforms for circuit construction was strengthened by further studies using the zebrafish embryo. In many model organisms, developing olfactory and optic nerve tracts and commissures containing scaffolds of pioneer axons are the predominant form of contact-mediated guidance structures in the developing nervous system (Ross *et al.*, 1992; Wilson *et al.*, 1990; Bastiani *et al.*, 1984; Eisen *et al.*, 1989; Gertler *et al.*, 1989; Kuwada, 1986; Raper *et al.*, 1984; Whitlock and Westerfield, 1998). But what are the guidance mechanisms directing the formation of these pioneer scaffolds?

Pioneering *Xenopus* retinal ganglion cells (Holt, 1989) and zebrafish motor neurons (Eisen *et al.*, 1989), form scaffolds for the fasciculation of succeeding axonal projections or "followers". Molecules known to be important in the formation of pioneer scaffolds include heparan sulfate proteoglycans (HSPG), chondroitin sulfate proteoglycans (CSPG), laminin and the intracellular signalling molecules phospholipase C-γ (PLC) and phosphoinositide 3-kinase (PI_3K) (Ishii *et al.*, 1992; Rajan and Denburg, 1997; Tisay and Key, 1999; Treloar *et al.*, 1997). Cell contacts mediated by the neural cell adhesion family of molecules (N-CAMs) are crucial for target recognition rather than tract formation in the mouse olfactory system (Treloar *et al.*, 1997). Earlier studies however, have shown that N-CAMs and L-1 are

temporally expressed throughout the developing olfactory system, important in the stabilisation of fascicles (or bundles of axons), especially between axons and olfactory ensheathing cells (OEC) suggesting a contextual relevance for N-CAMs (Dowsing *et al.*, 1997; Miragall *et al.*, 1989). The formation of corticothalamic fascicles and tracts in the developing rat is dependent on homotypic fasciculation, where cortical pioneers can facilitate fasciculation of future cortical cells rather than with thalamic projections (Bagnard *et al.*, 2001), enhancing the notion of tracts being scaffolded with similar cell types, although homotypic fasciculation is not a requirement for targeting in all tracts (Treloar *et al.*, 2002) (Sink and Whitington, 1991). Pioneering axons also interact with intermediate navigational checkpoints, or guidepost cells during early tract formation. Longitudinal pioneer axons in *Drosophila* form tracts by using trajectories largely defined by glia located lateral to the midline. The glia subsequently provide guidance and trophic support to following axons involved in midline crossing (Kinrade *et al.*, 2001).

Follower axons use molecular interactions with pioneer axon scaffolds and extracellular matrix proteins (ECMs) to find their targets. Cell contacts in the insect limb bud are mediated by adhesion molecules such as laminin, and are crucial for navigating past crucial choice points (Bonner and O'Connor, 2001). In the zebrafish, homophilic binding of secondary motor axons expressing DM-GRASP mediates fasciculation (Fashena and Westerfield, 1999), while in the vertebrate olfactory system, pioneering olfactory ensheathing cells mediate cell contacts via laminin, HSPG (Treloar *et al.*, 1996), and L-1 (Miragall *et al.*, 1989). Selective fasciculation of longitudinal tracts in mice is controlled by Pax6-regulated expression of r-cadherin, suggesting that transcriptional mechanisms mediate the ultimate shaping and termination of scaffold/tract formation (Andrews and Mastick, 2003).

These developmental studies have underscored the emerging idea that very early embryonic development is crucial for the succeeding layers, or waves of axons that build up and refine the final architecture of neural circuits. The demonstration of early scaffold development has reinforced the "Blueprint" or "Labelled Pathways" hypothesis of early scaffold formation which recognises that the embryonic neuroepithelium and primitive glial precursors define the primary neuronal pathways along which pioneering axons extend towards their cellular targets and that successive neurons make specific choices about which scaffolds they will extend upon (Raper *et al.*, 1983; Goodman *et al.*, 1983; Kuwada, 1986; Rajagopalan *et al.*, 2000; Raper *et al.*, 1984; Singer *et al.*, 1979). The question remains however, whether guidance cues found in the neuroepithelial environment are sufficiently textured or complex to explain the variety of scaffolding mechanisms seen so far. Indeed, examination of axon trajectories in transplanted *Xenopus* retinas has suggested multiple diffusible guidance cues, as well as specific positional cues also guide these axons (Harris, 1986, 1989).

1.5 Axon guidance mediated by diffusible guidance molecules

Pioneering work by Sperry showed that biochemical specificities and identities of neurons and not functional innervation was the overarching mechanism for appropriate axonal targeting in regenerating axons (Sperry, 1944; Sperry, 1963). While contact mediated axon guidance mechanisms utilise substrate dependent mechanisms to appropriately guide axons to their targets, what mechanisms would explain non-contact guidance? Diffusible gradients of guidance molecules have been proposed to act as long-range attractants in the development of the vertebrate spinal cord (Tessier-Lavigne

et al., 1988). Molecules and their receptors on growth cones able to perform such a long-range guidance role are relatively few in number during development and are arrayed in complex spatio-temporal expression patterns. In contrast, receptor heterogeneity, receptor complexes and signalling motifs that drive the diverse outcomes of simple receptor-ligand interactions would be expected to present an extremely complex signalling environment to axons performing navigational tasks. How do growth cones integrate and make sense of all these signals?

1.5.1 Neurotrophins

Neurotrophins are a family of proteins that comprise nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). They have a broad spectrum of biological functions in a variety of tissues (Kurihara *et al.*, 2003; Levi-Montalcini, 1987; Nassenstein *et al.*, 2004; Sariola, 2001; Tessarollo, 1998; Torres and Giráldez, 1998; Vega *et al.*, 2003). They have been well characterised in the developing nervous system, where they are actively transported to cell somata, initiating diverse cell-survival mechanisms (for review see Campenot and MacInnis, 2004).

Three types of neurotrophin receptors have been described so far. The high affinity tyrosine kinase (TyR) receptor TrKA is the receptor for NGF (Kaplan *et al.*, 1991), TrKB for BDNF and NT-4 (Fu *et al.*, 1999) and TrKC is the primary receptor for NT-3 (Lamballe *et al.*, 1991). TrK receptors are transmembrane tyrosine kinase receptors that serve as scaffolds for the recruitment of a variety of crucial intracellular signalling molecules such as PLC, PI_3K (Ming *et al.*, 1999) and RhoA (Yamashita *et al.*, 1999). The low affinity $p75^{ntr}$ receptor is common to all four neurotrophins (Rodriguez-Tébar

and Barde, 1988). The demonstrated interactions between $p75^{ntr}$ and RhoA firmly places the neurotrophins as important mediators of guidance signal transduction to the actin cytoskeleton (Gallo and Letourneau, 2000; Gehler *et al.*, 2004; He and Garcia, 2004; Yamashita *et al.*, 1999).

The first (and prototypic) neurotrophin, NGF, was first isolated 60 years ago and its neurotrophic effect was demonstrated 25 years later using ventricular injections of NGF into neonatal rats promoting attraction of sympathetic fibres towards the source of NGF (Levi-Montalcini, 1952; Menesini Chen *et al.*, 1978). Subsequent studies have confirmed its chemotrophic role in embryonic chick DRG neurons (Gundersen and Barrett, 1979; Letourneau, 1978). However, the lack of specificity within NGF-secreting targets and the observation that NGF mRNAs are transcribed after contact of target tissues by navigating axons suggests that NGF does not act as a long-range chemo-attractant but has important functions in cell survival and differentiation (Davies *et al.*, 1987; Lumsden and Davies, 1983; Snider, 1994).

Although much less abundant than NGF, BDNF mRNA is found predominantly in the CNS (Leibrock *et al.*, 1989) and has a crucial role in the formation of ocular dominance columns in the cat (Cabelli *et al.*, 1995; Cabelli *et al.*, 1997; Hata *et al.*, 2000) and the dendritic arborisation of retinal ganglion cells (RGC) (Lom *et al.*, 2002) and axon terminal density *in vivo* (Sanchez *et al.*, 2006). Furthermore, several studies have revealed a significant role for BDNF in the changes occurring at synapses during long-term potentiation (LTP) (Kossel *et al.*, 2001; for review see Poo, 2001) in mechanisms requiring BDNF in the synthesis of the immediate early gene product, Arc, (Yin *et al.*, 2002) mitogen activated protein kinase (MAPK) activation (Ying *et al.*, 2002), and PLC activation (Du and Poo, 2004). The

expression of BDNF is mediated by the calcium response elements, (CREB) and calcium-calmodulin kinase II (CaMKII), thus clearly implicating BDNF function with the influx of calcium (Jia *et al.*, 2007; Shieh and Ghosh, 1999).

Neurotrophins mediate significant motile activities in neurons (Dontchev and Letourneau, 2002) with BDNF having significant growth-promoting actions on filopodia (Gehler *et al.*, 2004) through RhoA and cofilin acivity (Gehler *et al.*, 2004). The regulation of growth cone motility by BDNF is calcium-dependent (Song *et al.*, 1997) and recent experiments have confirmed that transient receptor potential channels (TRPC) contribute to BDNF-induced calcium influx and are required for *in vitro* BDNF chemo-attraction of *Xenopus* spinal neurons and rat cerebellar cells (Amaral and Pozzo-Miller, 2007; Jia *et al.*, 2007; Li *et al.*, 2005a; Sossin and Barker, 2007).

1.5.2 Netrins

Netrins are secreted proteins with N-termini having laminin-like subunits including epidermal growth factor- (EGF) like repeats and unique C-termini. Netrins can act as long-range attractants or repellents. Netrin genes are highly conserved homologues of *C.elegans unc-6*, crucial for dorso-ventral migration of pioneer axons and mesodermal cells (Hedgecock *et al.*, 1990; Ishii *et al.*, 1992; McIntire *et al.*, 1992). A similar role for netrin in the patterning of the central axon pathways is conserved in other species. Two netrin genes in *Drosophila*, *netrinA* and *netrinB* are expressed by lateral neurons of the midline, mesodermal cells, glia and thoracic muscles, and are required for midline, commissure and motor axon guidance (Mitchell *et al.*, 1996). The vertebrate homologues, *netrin-1* and *netrin-2*, expressed by ventral spinal cord and floor plate cells of the rat (Kennedy *et al.*, 1994;

Kennedy *et al.*, 2006) and chick (Serafini *et al.*, 1996) are vital in attracting commissural axons in the spinal cord.

In addition to important functions in dorso-ventral patterning in the spinal cord, netrin-1 functions in other regions of the CNS including hippocampal, anterior and callosal commissure formation in the mouse (Serafini *et al.*, 1996). In the retina, netrins attract retinal ganglion cell (RGC) axons at short range to the optic disc (Deiner *et al.*, 1997) and in the forebrain where cortical projection neurons are guided to the internal capsule (Richards *et al.*, 1997). Peripheral projections including trochlear axons are guided dorsally by netrin-1 expressed by floor-plate neuroepithelium (Colamarino and Tessier-Lavigne, 1995).

Netrin receptors were initially characterised in studies on worm mutants, with *unc-5* affecting dorsal and *unc-40* affecting ventral migration (Chan *et al.*, 1996; Hedgecock *et al.*, 1990; Leung-Hagesteijn *et al.*, 1992). Homologues of *unc-40* in vertebrates, *deleted in colorectal cancer* (DCC) (Keino-Masu *et al.*, 1996), in *Drosophila*, *frazzled* (Kolodziej *et al.*, 1996) and of *unc-5*, UNC5H1, UNC5H2, and UNC5H3 (Ackerman *et al.*, 1997; Leonardo *et al.*, 1997) have similar effects. Another DCC-like netrin receptor, neogenin has been implicated in neural tube formation in the fish and the differentiation and development of non-neural tissues in the mouse (Keeling *et al.*, 1997; Mawdsley *et al.*, 2004). DCC mediates attraction to netrin while *unc-5* mediates netrin repulsion (Chan *et al.*, 1996; Hedgecock *et al.*, 1990; Kennedy *et al.*, 1994) alone, or in combination as a complex with DCC (Hong *et al.*, 1999). In the rat, *unc5h3* is important in the formation of corticospinal projections (Finger *et al.*, 2002) and in guidance of the trochlear and phrenic nerves (Burgess *et al.*, 2006).

In vitro, netrin attraction is contingent on appropriate levels of protein kinase A (PKA) activity, with low levels of cyclic adenosine monophosphate (cAMP) switching netrin attraction to repulsion (Ming *et al.*, 1997; Wu *et al.*, 2006). The role of cAMP in netrin signalling is further implicated in the cooperative signalling of DCC with the membrane-associated adenosine A2b receptor, a G-protein-coupled receptor that induces cAMP accumulation *in vitro* (Corset *et al.*, 2000). Downstream targets of netrin signalling include Enabled (ena), (Gitai *et al.*, 2003) and Rho GTPases/ cell death-10 (CED-10) /Rac-1 (Causeret *et al.*, 2004; Chang *et al.*, 2004), implicated in cytoskeletal rearrangements necessary for growth cone motility. Netrin-1 attraction is calcium-dependent with CaMKII, MAPK, calcineurin and nuclear factor of activated T-cells (NFAT) being major downstream targets of netrin-1 calcium signaling by way of calcium transients (Graef *et al.*, 2003; Hong *et al.*, 2000a; Tang and Kalil, 2005).

1.5.3 Semaphorins

The semaphorins consist of a large family of secreted and transmembrane glycoproteins. They fall into eight groups based on their domain organisation and species of origin, however they are all defined by a well-conserved, N-terminus, extracellular sema domain of approximately 500 amino acids. Vertebrate semaphorins are secreted (class 3) or membrane-bound (class 4-7) glycoproteins. Secreted semaphorins contain an Ig domain that is C-terminal to the sema domain, while the transmembrane family members may contain an Ig domain, a Type1 thrombospondin repeat or no other obvious motif N-terminal to the membrane. Class 2,3,4 and 7

semas have a single extracellular immunoglobulin-like domain while Class 5 semas posses seven thrombospondin domains.

Sema 1a (originally described as fasciclin IV) in the grasshopper limb bud epithelium (Kolodkin *et al.*, 1992), fly D-Sema1 and D-Sema2, and human Sema3a (Kolodkin *et al.*, 1993) are secreted proteins similar to chick collapsin-1, previously described as important in targeting of spinal and olfactory sensory axons (Kobayashi *et al.*, 1997; Luo *et al.*, 1993). Sema3a is chemo-repulsive to cortical rat axons but chemo-attractive to cortical apical dendrites (Bagnard *et al.*, 2000; Polleux *et al.*, 1998; Polleux *et al.*, 2000). Sema-Z1a, a zebrafish homolog of sema3a/collapsin-1 is expressed in axial muscles and guides posterior lateral line growth cones (Shoji *et al.*, 1998). Semas are crucial in olfactory bulb patterning (Cloutier *et al.*, 2004; Kobayashi *et al.*, 1997; Lattemann *et al.*, 2007; Renzi *et al.*, 2000; Schwarting *et al.*, 2000; Taniguchi *et al.*, 2003). Sema 3d guides RGC axons in the zebrafish (Liu *et al.*, 2004; Sakai and Halloran, 2006). Sema3a inhibits branching *in vivo* (Dent *et al.*, 2004). Sema5A induces collapse of rat RGC growth cones and inhibits RGC axon growth when presented as a substrate *in vitro* and in an optic nerve crush injury model (Goldberg *et al.*, 2004) but interacts with both CSPG and HSPG to be either attractive or repulsive respectively to habencular neurons in the rat diencephalon (Kantor *et al.*, 2004).

The plexin family of proteins are the pre-eminent sema receptors. Like the semas, plexin molecules include extracellular sema and Ig domains. Plexin molecules have been characterised in mice (Kameyama *et al.*, 1996), *Caenorhabditis elegans* (Fujii *et al.*, 2002) and *Drosophila* (Winberg *et al.*, 1998). In *Drosophila*, plexinA and plexinB interact with *Drosophila* sema1a

and 1b for crucial targeting and defasciculation of motor axons (Winberg *et al.*, 1998) and sema2a sensory axon guidance functions (Bates and Whitington, 2007). In addition, neuropilin-1 is the receptor for sema-3a (He and Tessier-Lavigne, 1997; Kolodkin *et al.*, 1997). While sema-2a binds to neuropilin-1 rather than directly to the plexins, neuropilin-1 forms a receptor complex with plexin-1, which serves the same signalling function as when it interacts directly with the membrane bound semas. To date, *C.elegans* and *Drosophila* neuropilin have not been described. These organisms do not express class 3 semas, however they express plexins suggesting that plexins are the most evolutionary conserved sema signalling moiety and that class 3 semas evolved much later, with neuropilin.

Semas can be either membrane bound or secreted, and be coupled with a multiplicity of potential receptors. How is this rich diversity of possible signalling combinations integrated into coordinated repulsion (or attraction) mechanisms? Specificity is accomplished by heteromeric combinations of receptor complexes. Neuropilins have no intrinsic intracellular enzymatic capacity and they function as ligand binding partners for sema3a-plexin1 co-receptor complexes, enhancing growth cone affinity to sema3a *in vitro* (Takahashi *et al.*, 1999). Downstream intracellular targets of sema signalling include Rac-1 and L1 mediated endocytosis of sema3a-neuropilin-plexin receptor complexes, RhoA activation, cGMP and Cdc42 signalling (Castellani *et al.*, 2004; Polleux *et al.*, 2000; Swiercz *et al.*, 2002; Toyofuku *et al.*, 2005), crucial components in dynamic actin rearrangements.

1.5.4 Ephs and ephrins

The Eph family of membrane-bound receptor tyrosine kinases, and their ligands, the ephrins, have been intensively studied in the developing avian visual system. The topographic mapping of retinal axons depends on gradients of Eph-A expression in retinal axons and complementary gradients of membrane bound ephrin-A ligands in the tectum. Axons expressing higher levels of EphA in a nasal to temporal retinal gradient are repulsed by an increasing anterior-posterior gradient of ephrin-A in the tectum (Brown *et al.*, 2000; Cheng *et al.*, 1995; Drescher *et al.*, 1995; Yates *et al.*, 2001). Other important spatial maps that utilise Eph/ephrin interactions include the hippocamposeptal system (Yue *et al.*, 2002), lateral motor column axons (Eberhart *et al.*, 2004; Kania and Jessell, 2003) and EphB/ephrinB3 in the mouse spinal cord (Kadison *et al.*, 2006).

The role of Ephs and ephrins in contact-mediated axon guidance generally is, however, seemingly paradoxical. Since many cells co-express both the EphA receptor and the cognate ephrin-A ligand, the question arises as to how overall axon guidance occurs in complex topographic targeting. Using chick motor axons, Marquardt *et al* (2005) showed that spatial segregation of EphA and ephrin-A molecules on growth cones enables both proteins to act independently in *trans* configurations, avoiding potentially confounding signalling repertoires.

1.5.5 Robos/Slits

Probably the best understood midline guidance system acts through the Slit/Roundabout (Robo) families of molecules. Originally described in a large scale mutagenesis screen for axon defects in *Drosophila* and a member of the immunoglobulin superfamily, Robo mediates midline crossing of commissural axons, binding to its large EGF-like ligand Slit which is expressed by glia at the mid-line (Battye *et al.*, 1999; Brose *et al.*, 1999; Kidd *et al.*, 1999; Kidd *et al.*, 1998b; Kidd *et al.*, 1998a; Rothberg *et al.*, 1990; Wang *et al.*, 1999). This interaction is dose dependent. Axons with low levels of Robo cross the midline, Robo-deficient axons cross the midline repeatedly and axons with Robo up-regulated or over-expressed never cross the midline. Axons that cross the midline up-regulate Robo expression to prevent further crossing. This dynamic expression pattern results in a series of spatially restricted commissures in the *Drosophila* ventral nerve cord (Kidd *et al.*, 1999; Kidd *et al.*, 1998b; Kidd *et al.*, 1998a; Seeger *et al.*, 1993).

Robo is highly conserved, with one *C.elegans* (Sax-3), 4 human, 3 *Drosophila* and 4 zebrafish genes (Kidd *et al.*, 1998b; Lee *et al.*, 2001; Zallen *et al.*, 1999; Zallen *et al.*, 1998). Robo function at the midline has been correlated with changes in growth cone dynamics in *Drosophila robo* mutants. Filopodial length, branch number and time spent near the midline are all elevated in null mutants, suggesting that constitutively active Robo acts to minimise filopodial protrusion, complexity and by inference, an axons' ability to navigate a choice-point (Murray and Whitington, 1999). Robo/Slit have similar growth cone dynamic functions outside the CNS with Robo preventing aberrant exploration by lateral cluster sensory axons in *Drosophila*, and in retinotectal and olfactory nerve projections in the

zebrafish (Hutson and Chien, 2002; Miyasaka *et al.*, 2005; Parsons *et al.*, 2003).

1.6 Neural activity and growth cone guidance

Conventional neuroscience dogma holds that the initial, developmentally regulated process of circuit wiring is activity independent, with activity only required for synaptic modification and dendritic arbor pruning (Hua and Smith, 2004; Katz and Shatz, 1996; Purves and Lichtman, 1980; Spitzer, 2006; Zhang and Poo, 2001; Zito and Svoboda, 2002). However, electrical excitability due to voltage-gated inward currents has been shown to have significant effects on motile growth cone behaviour *in vitro* and *in vivo*. Growth cones of *Helisoma* buccal neurons show a reversible halting of neurite extension and decrease in filopodial complexity with somatic current injection (Cohan and Kater, 1986). These effects are not consistent across populations of neurons, with varying temporal stimulation protocols producing variable growth cone effects (Cohan, 1990). Rat DRG axon outgrowth and growth cone morphology display considerably more sensitivity to phasic rather than tonic stimulation protocols (Fields *et al.*, 1990) while superior cervical ganglion (SCG) growth cones show little, if any sensitivity to electrical activity (Garyantes and Regehr, 1992).

Pathfinding mechanisms shown to be regulated by electrical activity vary considerably. Molecules such as growth associated protein-43 (GAP-43) are unchanged in mouse DRG neurons following electrical stimulation (Lin *et al.*, 1993), while L1 is down-regulated by patterned, low frequency stimulation (Itoh *et al.*, 1995). A variety of functional channels appear to be involved. Lateral geniculate neuron (LGN) projections in the embryonic cat visual

system require active voltage gated sodium (Na^+) channels to target appropriate corticothalamic projections (Catalano and Shatz, 1998). Conversely, RGC pathfinding and targeting in fish and *Xenopus* is tetrodotoxin (TTX) insensitive and 4-aminopyridine (4-AP) sensitive suggesting voltage gated potassium channels are important in the guidance of RGC axons (McFarlane and Pollock, 2000; Stuermer *et al.*, 1990). Neurotransmitters released due to localised areas of electrical activity may provide chemo-attractive cues to pathfinding axons. Such regulation of both growth cone motility and neurite extension and neurite elongation by neurotransmitters requires influx of extracellular calcium (Kater *et al.*, 1988; Mattson *et al.*, 1988). *In vitro*, acetylcholine receptors (AChR) are activated in a microgradient of ACh, leading to a small CaMKII-mediated rise in intracellular calcium concentration ($[Ca^{2+}]_i$) in *Xenopus* spinal neurons (Zheng *et al.*, 1994b).

1.7 Calcium and growth cone motility

1.7.1 Calcium is a crucial intracellular signalling molecule

Many activated cellular processes such as motility, membrane excitability, vesicle trafficking and apoptosis are initiated when $[Ca^{2+}]_i$ rises above resting or basal levels. In neurons, transient increases in $[Ca^{2+}]_i$ are thought to trigger neurotransmitter release and to modulate axonal transport, energy metabolism and growth cone motility (Smith *et al.*, 1983). The various effects attributed to one single, signalling ion are seemingly paradoxical. How can one signal mediate such a variety of processes and secondly, what, if any, are the overarching mechanisms and/or molecules that prevent uncontrolled cross-talk between the down-stream pathways? The answer

lies in the versatility, speed and spatio-temporal patterning of many calcium signalling processes. Many crucial calcium pathways are modularised and can be reassembled to create specific, reproducible biological effects (Berridge *et al.*, 2000). Neurons have developed many calcium signalling repertoires. For example, calcium is crucial for most pre and post-synaptic signalling. Presynaptic voltage-gated calcium channels orchestrate synaptic vesicle trafficking and release, and post-synaptically, calcium influx via receptor-operated channels propagates action potentials, activates cytoskeletal rearrangements and alters gene transcription. Much is known about calcium-specific signalling processes. However, much less is known about the calcium homeostatic mechanisms in neurons, especially those operating in motile growth cones.

1.7.2 Global calcium dynamics regulate protrusion of growth cones and neurite extension

The crucial role for calcium in mediating motile events in neuronal growth cones is now widely accepted. Intracellular calcium fluctuations are directly correlated with motile changes in the growth cone. Early *in vitro* experiments with *Helisoma* neurons showed that $[Ca^{2+}]_i$ is a molecular correlate of growth cone motility in response to electrical stimulation and receptor activation (Kater *et al.*, 1988; Kater and Mills, 1991; Mattson and Kater, 1987; Torreano and Cohan, 1997). Growth cone filopodia are strategically placed to initially detect and potentially transduce specific responses to guidance cues, while the growth cone central area amplifies and integrates these signals due to the participation of calcium stores (Davenport *et al.*, 1996; Ross *et al.*, 1989).

Chemotropic responses seen in the slime mould *Dictostelium* are correlated with changes in calcium concentrations. The calcium signal is not distributed homogeneously throughout the cell, but is higher in regions where active migration does not occur, or where the cell is resting (Yumura *et al.*, 1996). In *Helisoma*, the level of motile activity of growth cones varies with $[Ca^{2+}]_i$. Increases in $[Ca^{2+}]_i$ are prominent in all areas of the cell but more prominent in distal filopodial tips. Indeed the growth cone is the focus of calcium activity in the cell (Mattson and Kater, 1987; Torreano and Cohan, 1997). Abrupt increases in $[Ca^{2+}]_i$ halt protrusion or initiate collapse, while decreases to a suboptimal level may have varying effects on filopodial protrusion and neurite elongation, again implicating a specific $[Ca^{2+}]_i$ for appropriate growth (Cohan *et al.*, 1987; Kater *et al.*, 1988; Mattson and Kater, 1987).

A picture is emerging that $[Ca^{2+}]_i$ is a key regulator of motile and signalling states of growth cones, with actively growing or extending growth cones across a wide variety of model systems having significantly higher $[Ca^{2+}]_i$ than resting, or stable growth cones. Significantly, conditions that actively inhibit outgrowth, such as treatment with serotonin, can lead to much higher $[Ca^{2+}]_i$ (Davenport *et al.*, 1996; Komuro and Rakic, 1996; Mattson and Kater, 1987; Mattson *et al.*, 1988; Torreano and Cohan, 1997; Ziv and Spira, 1997). These key functions of $[Ca^{2+}]_i$ led to the formulation of the "setpoint hypothesis" that states,

> *"... if [intracellular] calcium falls below an optimal level, or rises significantly above it, growth cone motility and neurite outgrowth are inhibited..."* (Kater *et al.*, 1988; Kater and Mills, 1991)

Up to this time, researchers had concentrated on global effects of growth cone motility, with the behavioural readout being simple protrusion, stopping or growth cone collapse, and no understanding of the physiological or cytoskeletal underpinnings of these behaviours. In a series of technically challenging and innovative experiments using chick DRG neurons, Lankford and Letourneau (1989) correlated changes in growth cone shape and motile behaviour with perturbations in intracellular and extracellular calcium levels. Subsequent ultrastructural analysis of growth cones showed profound disturbances in actin filament stabilisation at the growth cone periphery. These observations suggested a crucial role for calcium in actin stability and supported a model of continuous actin assembly and disassembly as fundamental to growth cone motility.

1.7.3 Downstream targets of calcium signalling

1.7.3.1 Calcium/calmodulin-dependent protein kinase II and calcineurin

Like all protein kinases, CaMKII possesses a catalytic subunit that phosphorylates protein side-chains resulting in a conformational change affecting protein functions such as enzyme activity, cellular location, or association with other proteins. CaMKII has been intensely studied for its role in synaptic plasticity. It is required for long term potentiation of synaptic transmission in the hippocampus, while mice deficient in CaMKII exhibit learning defects (Malinow *et al.*, 1989; Silva *et al.*, 1992). The role of CaMKII in structural plasticity has also been the focus of much research, and is particularly relevant to the field of axon guidance. Post-synaptic expression of CaMKII in *Xenopus* tectal cells acts to reduce the complexity of axon

arbors *in vivo*. The exact mechanism of CaMKII action in this process is unclear, however premature expression of CaMKII in developing *Xenopus* tectal cells reduces dendritic branching and axon growth rates, suggesting that CaMKII limits and stabilises dendritic differentiation (Wu and Cline H. T., 1998; Zou and Cline H. T., 1996).

Calcineurin (CaN) is a serine- and threonine-specific protein phosphatase, highly conserved in all eukaryotes. It functions in the translocation of nuclear factor of activated T cell (NFAT) proteins by processing NFAT subunits in the cytosol, allowing subsequent translocation to the nucleus and assembly into transcriptional complexes. It is unique among phosphatases for its ability to sense calcium through its activation by calmodulin, linking transcription temporally to processes such as activity-induced calcium release in neurons (for review see Aramburu *et al.*, 2004). CaN functions in growth cone extension and filopodial retraction in mechanisms that are NFAT dependent (Graef *et al.*, 2003; Groth and Mermelstein, 2003; Lautermilch and Spitzer, 2000b). More recently, however, CaN and CaMKII have been shown to cooperate as a molecular switch in *Xenopus* growth cones. This molecular switch is sensitive to basal levels of $[Ca^{2+}]_i$ with shallow $[Ca^{2+}]_i$ gradients activating CaN and subsequent repulsive growth cone turning and normal to deep $[Ca^{2+}]_i$ signalling gradients activating CaMKII-mediated attraction (Wen *et al.*, 2004). A more detailed description of this molecular switch is presented in Chapter 4.

1.7.3.2 Cyclic adenosine monophosphate (cAMP)

cAMP is an important cytosolic second messenger involved in many cellular responses to extracellular signalling. A variety of growth cone functions

have been shown to be mediated by cAMP, including suppression of neurite outgrowth (Mattson *et al.*, 1988) and mediating attractive turning (Lohof *et al.*, 1992; Ming *et al.*, 1997). Perhaps its most relevant function in the current discussion is its role in *Xenopus* spinal neuron responses to netrin-1. Competitive, non-functional analogues of cAMP or inhibitors of PKA convert netrin-1 attraction to repulsion *in vitro* (Nishiyama *et al.*, 2003). As yet, there has been no direct evidence that this signalling pathway performs any function in growth cone guidance *in vivo.* Interestingly, recent studies by Wen *et al* (2004) elucidating the function of the CaMKII/CaN molecular switch, have revealed a negative regulation of the CaN repulsive pathway by cAMP/PKA. Clearly, this result places cAMP downstream as an effector of calcium-dependent signalling at the growth cone.

1.7.3.3 Cytoskeletal rearrangements

For some time, actin polymerisation and depolymerisation dynamics at the filopodial tips in cooperation with reorientation of stable microtubule bundles in the preferred direction of protrusion have been shown to underlie growth cone motility (Lin *et al.*, 1994; Mitchison and Kirschner, 1988). It is known that regulation of growth cone calcium is correlated with the stability of actin networks in motile growth cones (Lankford and Letourneau, 1989), however the exact nature of the role for calcium as a second messenger in the mechanisms controlling the cytoskeleton were unclear. Signalling pathways that directly regulate the stability of actin networks and microtubule dynamics have been well described in non-neuronal cells (for review see Burridge and Wennerberg, 2004). The master regulators of the actin cytoskeleton are the Rho family (including Cdc42) of small GTPases which are activated by binding to GTP and subsequent regulation of downstream effectors such as

the actin binding protein, cofilin (Aizawa *et al.*, 2001; Kuhn *et al.*, 2000) and the calcium/calmodulin-regulated scaffolding protein, IQ motif containing GTPase activating protein (IQGAP-1) (Kholmanskikh *et al.*, 2006). IQGAP-1 binds and stabilises GTP-bound Cdc-42 in its active form, mediating coupling of Cdc-42/Rac1 to actin filaments and ultimately polymerisation. This mechanism would directly link changes in cytosolic calcium with cytoskeletal rearrangements and motility.

1.7.3.4 Protein translation at the growth cone

The localisation of translational machinery in dendrites and growth cones has been well established (for reviews see Steward, 1997, 2002 a,b). The requirement for protein synthesis in growth cone motility was demonstrated by Campbell *et al* (2001) who showed local and rapid protein synthesis was necessary for attractive turning towards netrin-1 and repulsion from sema3A. These data described a paradoxical situation in the growth cone where translation regulated both attraction and repulsion. Mechanisms demonstrating local control of such translational machinery were still unclear, however, the integration of protein synthetic mechanisms into existing pathways of growth cone motility, such as calcium dependence, were described by Ming *et al* (2002). In microgradients of increasing concentrations of BDNF or netrin-1, *Xenopus* spinal growth cones demonstrate adaptive behaviour that desensitises the growth cone to the guidance cues. This is followed by a period of resensitisation to the cue which is protein synthesis dependent. These data support the emerging view of growth cones recalibrating their guidance receptor repertoire to suit specific long-range and intermediate navigational needs (Brittis *et al.*, 2002). A clearer picture is also emerging as to how translational machinery may

bias directional stability of the growth cone, hence regulating steering. The asymmetric localisation of β-actin mRNA is crucial to the attractive responses of *Xenopus* spinal neurons to the calcium-dependent guidance cues BDNF and netrin-1 (Leung *et al.*, 2006; Yao *et al.*, 2006). These dynamic translational processes would compliment and support calcium dynamics at the growth cone by providing signalling machinery for appropriate motile changes.

1.7.4 Asymmetric calcium is a correlate of guidance

By elevating $[Ca^{2+}]_i$ in spatially restricted areas of the growth cone, directed motility and protrusion can be reproducibly initiated in *Xenopus* spinal growth cones. Significantly, opposite responses can be initiated if baseline $[Ca^{2+}]_i$ is reduced (Zheng, 2000). The physiological relevance of this behaviour has been confirmed by experiments showing that calcium signalling is a molecular correlate of netrin-1-induced attraction *in vitro* (Hong *et al.*, 2000). *Xenopus* spinal growth cones are attracted to micr-gradients of netrin-1, and will alter their trajectories to directly target its source. These *in- vitro* attractive responses can be reversed when baseline $[Ca^{++}]_i$ levels are reduced by lowering extracellular calcium concentrations. Non-selective blocking of calcium influx or release of store calcium reverses *Xenopus* spinal neuron attractive turning, suggesting that calcium influx, from stores or membrane receptors is required for attractive turning (Hong *et al.*, 2000). These experiments confirm that calcium-dependent growth cone motile events have crucial, physiological relevance, and they support the developing notion of an intracellular calcium set-point or optimal level of $[Ca^{2+}]_i$ being necessary for appropriate motile responses of growth cones *in vitro*.

1.7.5 Shaping intracellular calcium: spikes, waves and transients

Periodic, spontaneous and transient calcium fluxes in growth cones have been observed in chick (Gomez *et al.*, 1995; Lohmann *et al.*, 2005) amphibian, (Gomez and Spitzer, 1999; Gomez *et al.*, 2001; Robles *et al.*, 2003) *Aplysia* (Ziv and Spira, 1997), hamster (Tang *et al.*, 2003), mouse (Jasoni *et al.*, 2007; Komuro and Rakic, 1996) and rat (Redmond *et al.*, 2002) neurons. A variety of physiological actions have been attributed to these events including mediating axon branching in response to netrin-1 signalling (Tang and Kalil, 2005), activation of calpain proteolysis and subsequent filopodial stability and growth cone turning (Robles *et al.*, 2003) and the regulation of calcineurin (Lautermilch and Spitzer, 2000), Effects of spontaneous events in growth cones include reduction in filopodial motility, slowing of neurite outgrowth or pausing and enhancement of turning when expressed asymmetrically.

The exact physiological nature of transient "events" is unclear. To varying degrees, the demonstration of these phenomena has paralleled the ability of microscopic techniques to resolve these periodicities on a spatial and temporal level. What appears to be consistent though is the consensus that all of these events originate from as yet unidentified channels on the cell membrane, since blocking known voltage-gated calcium channels only partially abolishes the propagation of these events (Molnar and Blakemore, 1995). There is wide variation in the nature, frequency and duration of transients in the literature, depending to a large extent on the model system and imaging protocols. The frequency of transient "spikes" and "waves" in *Xenopus* spinal neurons has been estimated at 3-10 events/hr *in vitro* (Gu

and Spitzer, 1995) (Gomez *et al.*, 2001). Elegant *in vivo* experiments using Rohon-Beard neurons in the intact *Xenopus* spinal cord showed similar (10 events/hr) frequencies in whole growth cones *in vitro* (Gomez and Spitzer, 1999). These studies in amphibians consistently show an inverse correlation of event frequency (not amplitude) with the rate of neurite outgrowth, and no demonstrable participation by intracellular stores, the absence of signal amplification and a general involvement of non-voltage gated membrane channels. Hamster cortical neurons, however, show a different picture. In large, paused, growth cones the transient frequencies were comparable (1-3 events/min), however they were sensitive to blockade of traditional L-type voltage gated calcium channels by nimodipine and nifedipine. Transients were partially blocked by thapsigargin indicating considerable heterogeneity in the population of neurons and a multiplicity of mechanisms contributing to generation of transient events (Tang *et al.*, 2003). Subsequent experiments by the same group using identical neurons showed that transient events associated with netrin-1 signalling involved the release of calcium from stores (Tang and Kalil, 2005).

The exact mechanisms by which transients exert their effects are unclear. In particular, there is little or no evidence to correlate transient calcium activity with global spatio-temporal changes in growth cone calcium concentration and specific motile changes. Demonstration of such a role would substantially confirm transients as crucial regulators of the growth cone set-point. Transient calcium events, however, can be interpreted as quantal instructional signals, probably initiated at filopodia but integrated globally throughout the growth cone, and ultimately leading to regulation of downstream targets of calcium signalling (Gomez *et al.*, 2001).

1.8 Mechanisms of calcium entry and storage

1.8.1 The endoplasmic reticulum is a key calcium buffer

The neuronal endoplasmic reticulum (ER) is a continuous, elaborate membranous structure responsible for a variety of cellular functions such as protein synthesis and calcium storage and release (Ross *et al.*, 1989). The membrane is continuous throughout the soma, dendrites and axonal growth cones and can be closely apposed (20-80 nm) to the cell membrane (Terasaki *et al.*, 1994). Inositol triphosphate (IP_3R) and the ryanodine (RyR) receptors are important ER constituents, responsible for regenerative calcium release, making the ER a crucial component in $[Ca^{2+}]_i$ homeostasis. While there is known variability in the subcellular distribution of IP_3R and RyR in avian cerebellar Purkinje neurons (Walton *et al.*, 1991), little is known of the relative amounts of IP_3R and RyR in neuronal growth cones (Sharp *et al.*, 1993). A conserved molecular strategy appears to concentrate calcium release proteins, for example chromogranin B which enhances calcium release from IP_3R in ER "hot spots", while calcium re-uptake elements such as sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA2) and plasma membrane calcium ATPases (PMCA1) calcium pumps show an even distribution throughout the cell (Jacob *et al.*, 2005). Receptor-activated release of calcium via the IP_3R begins with the activation of membrane receptors, followed by the activation of PLC and the subsequent enzymatic breakdown of phosphatidylinositol bisphosphate (PIP_2) into IP_3 which is the ligand for the IP_3 receptor. Binding of IP_3 to the IP_3R sensitises the receptor to lumenal and/or cytosolic calcium changes, which in turn, enables calcium release from internal stores. This release of calcium triggers a local, regenerative calcium wave as a result of continuous calcium induced calcium

release from adjacent IP_3R at the wave front. When a modest amount of IP_3 is bound to the receptor, a rapidly increasing calcium concentration surrounding the wave front auto-inhibits any further calcium release from the receptor, thus preventing a self-propagating, runaway calcium wave (Adkins and Taylor, 1999). This mechanism is modified, however, when low amounts of IP_3-bound IP_3R are present, that is, when a smaller stimulus is present. In this case, unbound receptors are sensitive to calcium and not inhibited. These mechanisms can be seen as fundamental to the generation of "graded" waves of calcium signals, or the initiation of cytosolic calcium spikes versus large waves (Berridge, 1998; Koizumi *et al.*, 1999). As yet, there is no clear understanding of the relative contributions of the IP_3R and the RyR to this process.

1.8.2 Receptor-mediated calcium entry

1.8.2.1 Voltage-gated calcium channels (VGCC)

Voltage gated calcium channels (VGCCs) are a set of plasma membrane proteins that respond to membrane potential depolarization by opening a calcium selective pore, thus allowing the entry of free calcium into the cytosol. While electrical activity has been implicated in growth cone guidance and growth cone morphology (see 1.6), the role of voltage-gated calcium channels (VGCCs) in these behaviours is not well understood. Growth cone calcium transients responsible for growth cone stalling in chick sensory neuron growth cones are not mediated by either L,N,T or P-type calcium channels but are sensitive to caffeine, suggesting the involvement of intracellular calcium stores via RyR (Gomez *et al.*, 1995). Similar effects of transient calcium activity on growth cone extension has been observed in

hamster cortical neurons, however these spontaneous calcium events were of a higher frequency and sensitive to blockade of L-type calcium channels with nifedipine (Tang *et al.*, 2003) suggesting regional differences in voltage-gated calcium channel expression and distribution may have a role in the effect of calcium transients on growth cone advance.

1.8.2.2 Transient Receptor Potential (canonical) (TRPC) channels

Originally characterised as a *Drosophila* gene required for signal transduction in the eye (Clapham, 1996), the TRP superfamily of genes encode a wide variety of channels that play crucial roles in biological processes (for review see Montell *et al.*, 2002). TRP channels have well described functions in transduction of sensory stimuli, such as heat, cold and pain in the peripheral nervous system (Bautista *et al.*, 2006; Chen *et al.*, 2006; McKemy *et al.*, 2002; Montell *et al.*, 2002; Patwardhan *et al.*, 2006). The TRPC family are highly enriched in the mammalian brain (Strübing *et al.*, 2001) and are activated by receptor-mediated signal transduction pathways such as tyrosine kinase activated PLC and subsequent hydrolysis of PIP_2. The hydrolysis of PIP_2 releases the lipophilic second messenger diacylglycerol (DAG) and soluble IP_3 which activates the IP_3 receptor on the ER and subsequent release of calcium into the cytosol. Exactly how the activation of TRPC by receptor-mediated mechanisms is translated into their hypothesised store-release functions is not clear at the moment. All TRPC channels have transmembrane polypeptide subunits that assemble to form homotetramers (for review see Montell *et al.*, 2002) however heteromeric combinations of TRPC4 and TRPC5, TRPC1 and TRPC5 or TRPC1 and TRPC4 (Strübing *et al.*, 2001, 2003) have been demonstrated indirectly *in vivo* and have been shown to have differing physiological properties to their

homomeric forms when expressed *in vitro*. Recent work by Yuan *et al* (2007) has led to the hypothesis that Stim1 regulation of TRPC hetero-multimerization provides a link between store-depletion and channel activation.

In growth cones, vesicle-borne TRPC5 is dynamically redistributed from the cytosol and inserted into the plasma membrane following EGF-stimulated PLC activation. The effect of this translocation was to significantly promote neurite length and complexity in rat hippocampal neurons (Bezzerides *et al.*, 2004; Greka *et al.*, 2003). In non-neuronal cells *in vitro,* TRPC3 is translocated to the cell membrane and functionally gated in a process mediated by the formation of a Homer1b/c-TRPC3-IP_3 complex and the activation of IP_3R by IP_3 (Kim *et al.*, 2006). Further indications of the important calcium signalling role of TRPC channels in growth cones emerged from studies undertaken by several groups examining the role of TRPC channels and calcium homeostasis in growth cone motility. TRPC channel function is crucial for the appropriate motile responses of growth cones to netrin-1 (Wang and Poo, 2005) and BDNF (Li *et al.*, 2005; Shim *et al.*, 2005)

1.8.3 Mechanisms of calcium flux

Cells increase $[Ca^{2+}]_i$ in two ways: by release of calcium from intracellular stores or by allowing calcium to cross the cell membrane through the action of calcium channels. The amount of calcium available from the ER is limited. Therefore receptor-mediated calcium influx through channels in the plasma membrane drives most cellular responses. A major route of extracellular calcium into the cell is via store-operated channels (SOC), the archetypal

calcium release-activated calcium channel (CRAC) (Hoth and Penner, 1992). This process is characterised by an initial rise in $[Ca^{2+}]_i$ through emptying of intracellular calcium stores (via activation of the IP_3R or RyR) followed by a sustained influx of extracellular calcium through channels that are distinct from voltage-gated calcium channels (Putney, 1986). This process, although mechanistically simple, has proved difficult to elucidate experimentally. Membrane proteins such as TRPC have been thought of as having SOC functions (for review see Beech, 2005), although there is conflicting experimental evidence as to whether TRPC are true SOC. Recently however, a new group of proteins have been shown to fulfil all the requirements of CRAC channels. Using *Drosophila* S2 cells, HeLa and T-lymphocytes, stromal interaction molecule1 (Stim1) was found to be an ER calcium sensor (Roos *et al.*, 2005) that interacts directly with a protein on the cell membrane, exhibiting all the prerequisite electrophysiological properties of a CRAC channel, Orai1 (Feske *et al.*, 2006). Elegant experiments utilising total internal reflection fluorescence (TIRF) microscopy showed that Stim1 and Orai1 are translocated in a coordinated manner upon store depletion to form closely apposed complexes between the plasma membrane and the ER. However, there is still some uncertainty as to whether a Stim1/Orai1 complex actually forms a calcium channel (Clapham, 2007a; for reviews see Clapham, 2007b; Luik *et al.*, 2006). Recent work has demonstrated *in vitro* activation of TRPC by Stim1 in HEK cells (Yuan *et al.*, 2007). This suggests that although TRPC may not exhibit all the electro-physiological properties of CRAC channels, TRPC may indeed function co-operatively as SOC with other CRAC-like channels, possibly using a common ER calcium sensor such as Stim1.

1.8.4 Spatially restricted calcium signalling

Localised changes in growth cone $[Ca^{2+}]_i$ have been observed experimentally using specific, fluorescently labelled calcium indicators (Gomez *et al.*, 2001; Gomez *et al.*, 1995; Henley and Poo, 2004; Zheng *et al.*, 1994; Zheng *et al.*, 1996). The precise nature of the mechanisms that couple these changes in $[Ca^{2+}]_i$ to motile changes that ultimately lead to pathfinding, however, are unclear. A combination of cytoplasmic calcium pumps, buffers and ER exchangers would be predicted to shape, modulate and even attenuate the exact spatial profile of a calcium signal. Another potentially confounding aspect may be the extent to which signalling occurs in spatially restricted areas of the cell membrane such as growth cone filopodia (Davenport *et al.*, 1993; Gomez *et al.*, 1995; Gu and Spitzer, 1995). Contemporary calcium imaging techniques may therefore be incapable of adequately resolving the true nature of calcium signals in the growth cone. Significantly, one probable role of such spatially restricted signalling in the context of growth cone motility, would be to restrict unwanted downstream cross-talk between possibly opposing pathways. It is interesting to note that spatially restricted calcium signalling is a feature of dendritic spine structure, organisation and function. Receptor organisation (including clustering) is regulated by scaffolding proteins that physically couple and restrain the signalling components between the plasma membrane and the ER. Important molecules with demonstrated scaffolding functions include the Homer and Shank protein families (Guthrie *et al.*, 1991; Hayashi *et al.*, 2006; Müller and Connor, 1991; Roche *et al.*, 1999; Sala *et al.*, 2001; Tadokoro *et al.*, 1999). Homer proteins can physically bind and couple group1 metabotropic receptors to the IP_3R, RyR and TRPC channels (Kato *et al.*, 1998; Tu *et al.*, 1999; Tu *et al.*, 1998; Yuan *et al.*, 2003). Significantly,

Homer is crucial for pathfinding and targeting of *Xenopus* tectal cells *in vivo* (Foa *et al.*, 2001). The question therefore arises: can intracellular scaffolding complexes transduce or regulate extracellular guidance cues and subsequent calcium signalling in the growth cone?

1.9 Homer and axon guidance

1.9.1 Homer protein

Homer proteins (also known as vesl, CPG-22, cupidin or PSD-45) are best known as molecules which facilitate signalling at the post synaptic density. Homer was initially characterised from two-hybrid screens for genes up-regulated after chemically-induced seizure in rats (Brakeman *et al.*, 1997; Kato *et al.*, 1998; Kato *et al.*, 1997; Nedivi *et al.*, 1993; Shiraishi *et al.*, 1999; Tadokoro *et al.*, 1999; Tu *et al.*, 1998; Xiao *et al.*, 1998). This activity-inducible, or "short" form Homer 1a, has been demonstrated in rodents (Brakeman *et al.*, 1997). In mammals there are 3 homer genes which undergo alternative splicing to produce multiple functional isoforms. Homer proteins are highly conserved across species including human, rat, mouse, Drosophila and Xenopus (Diagana *et al.*, 2002; Foa *et al.*, 2001; Xiao *et al.*, 1998). To date however, only the homer 1 gene has been described in non-mammalian species.

Homer proteins are defined by their N-terminal EVH1 (Enabled Vasodilator-stimulated phosphoprotein Homology) domain. The Homer EVH1 domain facilitates binding to other proteins, such as metabotropic glutamate receptors (mGluR), IP_3R, RyR, TRPC and other anchoring proteins such as Shank (Kato *et al.*, 1998; Tu *et al.*, 1999; Tu *et al.*, 1998; Yuan *et al.*, 2003).

The constitutively expressed, non-inducible, "long-form" Homer proteins (Homer 1b, 1c, 2a, 2b and 3) also contain a C-terminal coil-coiled (C-C) domain through which they dimerise homophilically. Homer 1a lacks a C-C domain but has a fully functional EVH1 domain. Consequently it has been described as a dominant negative form of Homer (Tu *et al.*, 1998).

Homer mutations have been placed in a variety of biochemical pathways associated with addiction (Berman *et al.*, 2004; de Bartolomeis and Iasevoli, 2003; Fourgeaud, 2005; Fourgeaud *et al.*, 2004; Ghasemzadeh *et al.*, 2003; Hashimoto *et al.*, 2004; Kane *et al.*, 2005; Swanson *et al.*, 2001; Szumlinski *et al.*, 2004; Szumlinski *et al.*, 2004; Urizar *et al.*, 2007). Homer has also been implicated in a variety of neurological syndromes such as CRASH and Fragile-X (Giuffrida *et al.*, 2005; Govek *et al.*, 2004), epilepsy (Klugmann *et al.*, 2005; Potschka *et al.*, 2002), stroke (Rickhag *et al.*, 2006) and neurodegenerative conditions including Parkinson's disease (Hubert and Smith, 2003) and chronic pathological pain (Tappe *et al.*, 2006). These diverse but clinically significant conditions would suggest that Homer is possibly a developmentally regulated protein, necessary for the formation and functioning of complex neural circuitry.

1.9.2 Homer at the PSD and growth cone

To date, Homer function at the growth cone has not been elcidated. However, Foa *et al* (2001) have shown that by expressing Homer1a or Homer1b/c in wild-type *Xenopus* tectal cells, significant pathfinding errors occurred in both commissure recognition and tectal targeting. Since both treatments would be expected to perturb constitutive Homer1b/c expression, this would suggest that a crucial level of Homer 1b/c was necessary for

accurate pathfinding and target-recognition in the developing *Xenopus* retino-tectal system. To find a prominent and important PSD scaffold protein in the growth cone would not be surprising. At the PSD, Homer functions with other scaffold proteins such as Shank and PSD-95 to regulate the function, distribution and signalling of mGluR and N-methyl D-aspartate (NMDA) receptors. Since both the PSD and the growth cone share a common suite of signalling machinery, such as neurotransmitter receptors, kinases, phosphatases and cytoskeletal regulatory proteins, it would not be surprising to find Homer playing an important role in both signalling environments.

1.9.3 Homer, calcium and synaptic plasticity

It has been postulated that Homer proteins regulate normal neuronal function and development by mediating cytosolic protein interactions following extracellular signaling. To date, long forms of Homer have been shown to have important functions in receptor clustering (Ango *et al.*, 2000; Ango *et al.*, 2002; Ciruela *et al.*, 2000; Roche *et al.*, 1999; Sala *et al.*, 2001; Tadokoro *et al.*, 1999; Usui *et al.*, 2003), axon pathfinding (Foa *et al.*, 2001) and dendritic remodelling (Sala *et al.*, 2001). Perhaps the most widely studied receptor interaction with Homer protein is the functional regulation of the metabotropic receptor by Homer. The metabotropic glutamate receptors are functionally and pharmacologically distinct from the ionotropic glutamate receptors. Group1 mGluR are coupled to G proteins and activate PLC, to form PIP_2 with subsequent activation of PKC and production of IP_3 to release store calcium. Homer protein dimers couple mGluR and IP_3R via an N-terminal EVHI domain. Homer 1a competes with constitutively expressed long-form Homer dimers to uncouple the Homer-mGluR complexes,

effectively attenuating glutamate-induced intracellular calcium release (Kammermeier and Worley, 2007; Kammermeier *et al.*, 2000; Tu *et al.*, 1998).

At excitatory synapses, changes in amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor transmission underpins the maintenance of synaptic strength, and mGluR modulation of AMPAR plasticity occurs through the induction of Homer1a via synaptic activity (Van Keuren-Jensen and Cline, 2006). These findings suggest a mechanism by which the activation history of a synapse is reflected in the expression and structural coupling of an activity-related protein such as Homer1a.

For the purposes of this thesis, and this point on, the term Homer will refer to the family of proteins encoded by the *homer1* gene. Since significant Homer1a expression has only been demonstrated in activity-induced tissues (Brakeman *et al.*, 1997), reference to Homer in the context of DRG neurons, unless stated otherwise, will refer to Homer1b and/or Homer1c. In the context of Homer morpholino knockdown, the term Homer morphant refers to cells transfected with Homer1 morpholino and displaying reduction of constitutive expression of Homer1b/c.

1.10 Hypothesis and aims of this thesis

This study poses questions that are fundamental to our understanding of the development of neural circuits. There is a body of evidence that suggests multiple, overlapping and cooperative signalling mechanisms are present in the growth cone to transduce guidance cues to the motile machinery. It is also known that the regulation of intracellular calcium plays a crucial role in

many of the processes necessary for appropriate motile responses of growth cones. The underlying mechanisms that transduce guidance cues and control the basal calcium level, or "set-point" at present remain unclear.

The study aims to provide evidence for a mechanism of signal transduction based on the hypothesis that Homer acts at the growth cone to facilitate the transduction of extracellular signals in a calcium-dependent manner.

This hypothesis will be tested under the following specific aims. Each specific aim will be addressed by an experimental chapter. The concluding chapter will discuss the findings of the work undertaken, their implication to the field of axon pathfinding and future directions. The specific aims are as follows:

1.10.1 Homer is a molecular correlate of sensory system development in the zebrafish

Homer has previously been shown to be crucial for axon pathfinding in the developing *Xenopus* sensory nervous system. If Homer is important in the formation of synaptic circuitry then it would be predicted to be present in a developmentally regulated manner. Experiments described in Chapter 2 will specifically ask whether Homer is developmentally regulated in the developing zebrafish embryo.

1.10.2 Motility of dorsal root ganglion (DRG) sensory neurons in an *in vitro* growth cone turning assay

If Homer is important in axon pathfinding, then it would be predicted to have crucial functions at the growth cone. To fully characterise the mechanism of Homer function at the growth cone, a cell culture approach will be used. This specific aim is addressed in Chapter 3. A relevant sensory model system will be assessed for its ability to respond to well-established guidance cues in an *in vitro* assay of growth cone motility. This system will then be used to address specific aim #3.

1.10.3 Homer signals through the operational state of a CaMKII-CaN molecular switch *in vitro.*

Motile responses of growth cones to extracellular guidance cues require the transduction of those signals to the intracellular cytoskeletal machinery. Homer function in pathfinding has previously been found to dose dependent, with a crucial level necessary for correct axonal trajectories in the *Xenopus* retino-tectal system. If Homer's function in growth cone guidance is to facilitate the transduction of guidance signals, then it would be predicted that reduced Homer expression would lead to perturbations in the motile responses to well established guidance cues. Chapter 4 describes experiments that will decrease the level of Homer1b/c and then uses these cells to assess any changes in their motile behaviour.

1.10.4 Homer knockdown alters the calcium dynamics of motile growth cones

It is established that calcium regulates many cellular processes, including growth cone motility, in a manner that is dependent on basal $[Ca^{2+}]_i$. If Homer does indeed function in axon pathfinding, does it do so by regulating $[Ca^{2+}]_i$? Chapter 5 presents experiments that will follow the course of calcium flux in motile growth cones with constitutive or reduced levels of Homer1. If Homer is required for these calcium-dependent functions then it would be predicted that motile events in cells with low expression of Homer1b/c would be significantly altered.

Chapter 2

Homer is a molecular correlate of sensory system development in the zebrafish

2.1 Introduction

The development of neural circuitry in the embryo can be seen as a continuum of successive signaling events, each molecular signal having a cumulative effect on the strength and unity of the final circuit. By studying the developmental biology of these emerging circuits, we can gain an understanding of the key molecules and mechanisms responsible for functional wiring in the brain. In an effort to establish whether Homer protein is an important guidance molecule *in vivo*, its expression was characterised in the developing zebrafish to ascertain whether Homer is present in a specific axon pathfinding spatio-temporal context. Much of this work has been previously published by the author (Gasperini and Foa, 2004).

The zebrafish is an excellent model in which to study the role of genes in the developing nervous system. The relatively short generation time (Westerfield, 1995), accessibility and transparency (Fishman *et al.*, 1997) of embryos in their yolk sacs, extensive array of axon pathfinding mutants (Karlstrom, 1996; Karlstrom *et al.*, 1997) and genomic information make the zebrafish a relevant vertebrate *in vitro* and *in vivo* experimental model (Key and Devine, 2003). The early scaffold formation and topography of projections in the embryonic brain has been well described (Devine and Key, 2003; Eisen, 1991; Hjorth and Key, 2001; Karlstrom, 1996; Karlstrom *et al.*, 1997; Kimmel *et al.*, 1995; Ross *et al.*, 1992b; Trevarrow *et al.*, 1990; Westerfield *et al.*, 1990; Wilson and Easter, 1991; Wilson *et al.*, 1990). In addition, techniques for the modification and alteration of gene expression such as morpholino-induced gene knockdown (Draper *et al.*, 2001; Scholpp and Brand, 2001), targeted

electroporation (Swartz *et al.*, 2001) and laser-induced gene expression (Halloran *et al.*, 2000) are well developed.

Two particularly well-described and accessible models in the fish are the olfactory and optic systems. This chapter will focus on the olfactory system, a biological system of particular relevance to an organism such as a zebrafish. The well-developed sense of olfaction in the zebrafish is vital to allow larvae and adult fish to find food, mate and escape predation. In general, fish have exquisitely sensitive olfactory thresholds, as evidenced by the eel's (*Anguilla anguilla*) astounding ability to detect phenylethyl alcohol at a level of 2.9×10^{-20} M (Little *et al.*, 1983). The zebrafish olfactory system parallels that of higher organisms in many ways. The olfactory apparatus develops as a placode or invagination of ectoderm. When raised under standard conditions at 28.5 ^{o}C, the development of olfactory sensory neurons (OSN) and pioneer neurons begins at approximately 17 hours post fertilization (hpf). Hatching subsequently occurs at approximately 48 hpf (Westerfield, 1995).

Axon scaffolds laid down by pioneer neurons form a basic guidance mechanism for the development of OSN connections into the developing forebrain. By 60 hpf, well developed axonal connections are established in the anterior and medial regions of the presumptive olfactory bulb, where they project to the first relays in the olfactory circuit, the glomeruli, in much the same way as has been described in mammalian systems (Baier and Korsching, 1994; Baier *et al.*, 1994; Byrd and Brunjes, 2001; Dynes and Ngai, 1998; Westerfield, 1995; Whitlock and Westerfield, 1998).

In the adult fish, the distal sensory organ or olfactory rosette, is composed of folds or lamellae of cellular elements arranged in a pseudo-stratified columnar

fashion. It is contiguous with the external environment via two nares allowing water and odorant molecules to pass over its epithelial surface. The OSN nuclei are typically located just above the basal epithelial surface. Cilia, microvilli and OSN dendrites are prominent structural features of the sensory epithelium, and being the first structures to be exposed to odorant molecules, are predicted to be the locus of odorant receptor localization (Hansen *et al.*, 2004; Hansen *et al.*, 2003; Menco *et al.*, 2003; Rhein and Cagan, 1983).

OSN axons project to the olfactory bulbs which are paired structures immediately rostral to the telencephalon. In the adult, the olfactory bulb is organized into 4 principal layers or laminae, namely the nerve, glomerular, mitral cell/plexiform and granule cell layers (Byrd and Brunjes, 1995; Byrd *et al.*, 1995). The OSN axons project into the olfactory bulb towards their specific targets in the glomerular layer, where they synapse with mitral and tufted cell dendrites. Odorant coding is relayed from the olfactory bulbs to higher brain centres (Baier *et al.*, 1994). This simple circuit provides a highly stereotypic model system to study *in vivo* growth cone trajectories in a biologically relevant context.

Homer proteins are highly conserved across species including human, rat, mouse, *Drosophila* and *Xenopus* (Diagana *et al.*, 2002; Foa *et al.*, 2001; Xiao *et al.*, 1998). To date however, only the homer 1 gene has been described in non-mammalian species (Diagana *et al.*, 2002; Foa *et al.*, 2005). In the rat, Homer is abundant in the cortex, hippocampus, cerebellum, heart, kidney and liver. In subcellular fractions, it is enriched in synaptoneurosomes (Xiao *et al.*, 1998). In *Xenopus*, it is abundant in neural tissue, particularly in sensory systems such as the optic tectum and retina (Foa *et al.*, 2005). Within the *Xenopus* nervous system, Homer expression displays a ubiquitous

cytoplasmic localization, prominent in the neuropil and at cellular junctions at the ventricular border (Foa *et al.*, 2005; Foa *et al.*, 2001). As yet, a zebrafish Homer gene family has not been fully characterised. Several zebrafish clones, however, reveal sequence identities and conserved structures similar to Homer genes in other organisms.

The aim of this study was to examine the ontogeny of Homer expression in the developing zebrafish embryo, particularly in sensory systems. Characterisation of such ontogeny provides significant clues regarding the role of Homer in the developing nervous system since the early development of the larval olfactory placode with the egress of sensory axons provides an excellent model system to study pathfinding. The experiments described in the chapter describe the developmentally regulated expression of Homer 1b/c in the zebrafish olfactory system using a well-characterised Homer 1b/c antibody. They show that Homer expression in the developing brain and olfactory system is correlated with behaviourally significant time-points, suggesting that Homer protein is crucial for functional sensory circuit development and may potentially underlie a crucial pathfinding role for Homer in olfactory circuit development.

2.2 Materials and methods

2.2.1 Zebrafish stock maintenance

Animals were obtained from an in-house wild-type breeding colony. Adult fish were housed in recirculating and aerated water with a 14 hr/10 hr light/dark cycle at 26-28 °C. Matings and egg collections were performed according to (Westerfield, 1995). Embryos and larvae were reared in glass fingerbowls at 28.5 °C and staged according to (Westerfield, 1995). All animals were deeply anaesthetised with tricaine methanesulphonate (0.05%, MS-222, Sigma, USA) before tissue removal or fixation. All animal procedures were approved by the University of Tasmania Animal Ethics Committee in accordance with the Australian National Health and Medical Research Council animal use guidelines.

2.2.2 Protein sample preparation

Dechorionated and de-yolked embryos, larval and adult tissue were rapidly homogenized in RIPA buffer: (50 mM Tris pH 7.4, 150 mM NaCl. 1 mM phenyl methane sulfonyl fluoride (PMSF, 1 mM), ethylenediamine tetra acetic acid disodium (1 mM, EDTA), aprotinin (5 µg/ml), leupeptin (5 µg/ml), 1% $^{v}/_{v}$ Triton X-100, 1% $^{v}/_{v}$ Na deoxycholate, 0.1% $^{v}/_{v}$ sodium dodecyl sulfate (SDS); all reagents from Sigma, USA).

2.2.3 Protein quantitation

Protein samples were assayed for total protein concentration using a commercial detergent-compatible total protein assay kit (D_C Protein Reagent Kit, Bio-Rad, CA, USA). Briefly, protein samples in RIPA buffer were diluted I in 5 with water then 25 µl were added to 125 µl of Reagent A followed by 1 ml of reagent B. Samples were mixed then incubated at room temperature for 15 min and absorbances measured spectrophotometrically at 750 nm. Unknown absorbances were compared to absorbances derived from a set of albumin standards (bovine albumin Type V, Sigma) assayed in the same protocol.

2.2.4 Protein electrophoresis and Western blotting

Exactly 30 µg total protein from selected tissues was separated on 10-12% polyacrylamide gel (SDS-PAGE) then electro-blotted for 2 hr onto 0.2 µm polyvinylidene difluoride (PVDF, Bio-Rad, CA, USA) membranes then blocked overnight in blocking solution (0.5% $^{v}/_{v}$ skin milk powder). Homer protein was probed with an anti-rabbit Homer 1b/c antibody (1:1000, Xiao *et al.*, 1998, a generous gift from Prof. Paul Worley, Johns Hopkins School of Medicine, Baltimore, MD, USA). Antibody conjugates were detected goat anti-rabbit-HRP (1:5000 Dako, USA) and ECL© chemiluminescence reagent (Pierce, IL, USA).

2.2.5 Immunohistochemistry

Dechorionated embryos, larvae or adult tissues were fixed overnight at 4°C in 4% v/v paraformaldehyde in 0.1M phosphate buffered saline, (PBS, pH 7.4), followed by infiltration with 30% sucrose in PBS at 4°C for 4-24 hours. Tissues were oriented and mounted in Tissue-Tek® embedding medium (Miles, USA) and rapidly frozen in liquid nitrogen. Cryosections (7-12 µm) were mounted on 3-aminopropyltriethoxysilane (APTS, Sigma, USA) treated slides, air dried at 22-24 °C for 2-4 hr and subsequently stored at –20 °C in a desiccator. After rehydration in PBS, sections were permeabilised and blocked in 0.4% $^{v}/_{v}$ Triton X-100, 5% $^{v}/_{v}$ normal goat serum in PBS for 1 hr at 22 °C, then incubated overnight at 4 °C in primary antisera: Homer 1b/c, 1:1000 (Xiao *et al.*, 1998); a dendritic marker, MAP2, 1:2000, (Chemicon, USA) and a specific zebrafish neuronal cell surface marker ZN-12, 1:250, (Trevarrow *et al.*, 1990) (Developmental Studies Hybridoma Bank, USA). Negative control slides were prepared by omitting primary antibodies and incubating sections with sera alone. All other experimental reagents and conditions were identical. Primary antibodies were detected with Alexa-Fluor 488 or 594 secondary antibodies (Molecular Probes, OR, USA). Images were acquired using an Axioplan 200 (Zeiss, Germany) microscope equipped with a Zeiss Apotome real-time image deconvolution module and Axiocam MR CCD camera or a Leica LD-82 microscope (Leica, Wetzlar, Germany) equipped with a MagnaFire CCD camera (Optronics, CA, USA). Images were imported into, and formatted with Photoshop CS3 and Illustrator CS3 (Adobe, CA, USA).

2.2.6 Genomic database searches

Using partial sequence from the highly conserved EVH1 domain from the rat, the UniProt Knowledgebase (European Bioinformatics Institute (EBI), Swiss Institute of Bioinformatics (SIB) and Protein Information Resource (PIR)) was queried. Several similar clones, including AAH77128.1 (BC077128) were recovered and then used in a Clustal W analysis for sequence similarity and domain recognition, comparing the putative zebrafish protein to *Xenopus*, rat, bovine, mouse and human proteins.

2.3 Results

2.3.1 Zebrafish genome contains a putative Homer 1b/c homologue

Analysis of sequence data (UniProt, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, USA) for H1b/c shows a high degree of homology between human, rat, mouse (Xiao *et al.*, 1998), bovine (NIH- Mammalian Gene Collection Project, 2005) and *Xenopus* (Foa *et al.*, 2005). Using the rodent sequence from the highly conserved EVH1 domain, a putative zebrafish homologue was extracted from embryonic zebrafish cDNA sequence data (Strausberg and others, 2002). Pair-wise lustal analysis across all Homer homologues showed an amino-acid identity of 70.6% and a similarity of 85%. (Fig 2.1). All proteins are similar in size with the only exception being zebrafish H1b/c with 2 small insertions: a 35 amino-acid residue insertion at the N-terminus immediately after the start codon, and a smaller 6 amino-acid residue insertion between the highly conserved WASP homology 1 domain (WH1, containing the conserved EVH1 motif), and the similarly highly conserved C-terminal coiled-coil domains (Fig 2.1). Significantly, the zebrafish sequence is identical with all other sequences at the WH1 (EVH1) domain, the region that defines all Homer proteins, and the C-terminal portion of the coiled-coil domain (Fig 2.1, 93.3% similar and 88.2% identical across species).

2.3.2 Homer 1b/c expression is developmentally regulated in zebrafish

As has been found previously in *Xenopus* tadpoles (Foa *et al.*, 2005), expression of Homer is developmentally regulated in zebrafish larvae and embryos. Western analysis was used to confirm the presence of a zebrafish homologue of Homer 1b/c with a polyclonal antibody directed against the 18 C-terminal residues of the coiled-coil domain of rat Homer 1b/c (Tu *et al.*, 1998). This antibody detected the predicted 47 kD band for Homer 1b/c by protein immunoblotting of adult zebrafish brain and larval tissues (Fig. 2.2). This antibody has been used previously in mammalian and *Xenopus* tissue and has been shown to detect only the long form of Homer and not the activity induced, short form, Homer 1a (Foa *et al.*, 2005; Xiao *et al.*, 1998). Expression was first detected at 24 hpf and continued to increase throughout larval development. Expression remained high in the adult zebrafish, particularly in the brain although it was also present in other tissues, such as skeletal muscle, as has been described in mammalian and *Xenopus* tissue (Foa *et al.*, 2005; Foa *et al.*, 2001; Xiao *et al.*, 1998).

2.3.3 Spatial and temporal distribution of Homer1b/c in the developing olfactory placode

Homer 1b/c–like immunoreactivity (Homer-IR) was prominent in the developing zebrafish olfactory placode from 24 hpf through to adulthood. As described previously, a well-defined and discernible olfactory placode is first obvious in 16-24 hpf larvae (Baier and Korsching, 1994; Baier *et al.*, 1994; Barth *et al.*, 1996; Byrd and Brunjes, 1995; Kimmel *et al.*, 1995; Westerfield, 1995). In horizontal sections of larvae, the first detection of specific Homer-IR

in the developing olfactory placode was at 24 hpf (Fig. 2.3 B). Expression was evident around the placode with prominent ectodermal staining surrounding the primitive external nares. This specific and striking expression pattern was highly suggestive of basement membrane localization, clearly defining the boundary of the olfactory placode. Basement membrane localization was still apparent at 36 and 48 hpf (Fig. 2.3 C&D), but was greatly diminished by 72 and 96 hpf (Fig.2.3 E&F). At 24 hpf, Homer-IR was not observed in either OSN or pioneer neuron populations. By 48 hpf, expression had increased and extended into more apical areas of the placode (Fig. 2.3 D, arrowhead). A punctate distribution of Homer-IR was also observed along the naso-temporal axis, suggestive of cell-cell junctions (Fig. 2.3 D, small arrows) as has been described previously in mammalian tissue (Shiraishi *et al.*, 1999). By 72 hpf there was a marked change in Homer-IR which included a conspicuous increase in intensity in the apical dendrites (Fig. 2.3 E, arrowhead). This trend continued in the 96 hpf larvae where the entire apical surface was intensely labelled, suggesting that Homer-IR is enriched in the dendritic knobs of OSN's (Fig. 2.3 F and 2.3 G, arrowhead). In addition to this dendritic labelling, perisomatic Homer-IR, potentially at cell-cell junctions, remained prominent along the naso-temporal axis of the placode (Fig. 2.3 E&G, small arrows).

2.3.4 Spatial distribution of Homer1b/c in the adult olfactory neuroepithelium.

Homer-IR was detected in horizontal sections of adult sensory epithelium. The zebrafish olfactory epithelium is organised as a pair of cup-shaped rosettes with lamellae attached to a stalk or central raphe. Previous studies in goldfish and zebrafish have identified OSN's in lamellar areas adjacent to the midline raphe whilst at the more distal lamellar areas, there is a preponderance of respiratory epithelial components (Barth *et al.*, 1996; Byrd and Brunjes, 1995; Hansen *et al.*, 2005; Hansen and Zeiske, 1993). Homer-IR in horizontal sections of adult olfactory epithelium revealed a similarity to the expression of Homer-IR in the developing larval olfactory placode. In addition to a basement membrane localization (Fig. 2.4 A, arrow), there was specific apical distribution of Homer-IR in OSN's (Fig. 2.4 b, arrows). Punctate perisomatic staining was also discernible throughout the epithelium (Fig 2.4 B, arrowheads). These data suggest that significant Homer expression is maintained within OSN dendrites in adult zebrafish and may be potentially important in odorant coding.

2.3.5 Spatial and temporal distribution of Homer1b/c in the developing olfactory bulb.

Homer-IR was detected in the developing zebrafish forebrain and olfactory glomeruli. OSN axons project into the olfactory bulb and terminate onto stereotypic glomeruli in much the same manner as mammals. Olfactory glomeruli first appear in the forebrain of the zebrafish by 72 hpf (Barth *et al.*, 1996; Dynes and Ngai, 1998; Hansen and Zeiske, 1993; Whitlock and Westerfield, 1998), however by 96 hpf, olfactory glomeruli have assumed a more stereotypic globular morphology. At this stage of development,

prominent Homer-IR puncta were observed in the glomeruli of 96 hpf larvae (Fig. 2.5 A). Staining of 96 hpf larvae for the dendritic marker MAP-2, revealed an elaborate meshwork of fine dendritic processes defining the characteristic spherical arbors of olfactory glomeruli (Fig. 2.5 B). The Homer-IR puncta prominent in the glomeruli were closely apposed to MAP-2 staining, suggesting a dendritic and potentially synaptic localization (Fig. 2.5 C). A similar expression pattern was revealed with 96 hpf larval sections double-immunostained with Zn-12, a zebrafish monoclonal antibody that detects neurons and processes (Trevarrow *et al.*, 1990), and Homer 1b/c (Fig. 2.6), confirming that Homer is indeed expressed in neurons within the olfactory glomeruli.

2.3.6 Spatial distribution of Homer1b/c in the adult olfactory bulb.

Homer-IR remains a prominent feature in the cytoarchitecture of the adult olfactory apparatus. Staining of adult olfactory bulb for Homer 1b/c and MAP-2 revealed a population of large, pyramidal, Homer-IR cells close to the border of the mitral cell/plexiform and granular cell layers (Fig. 2.7 A,D & E). The major dendrites of these cells projected directly into the glomerular layer (Fig. 2.7 D&E). The size, shape and location of these cells was consistent with that of mitral cells. Closer examination revealed discrete puncta of Homer-IR closely apposed to MAP-2 positive puncta in the glomerular layer (Fig. 2.7 E, arrowheads). This pattern was similar to that seen in the sections of 96 hpf larval forebrain.

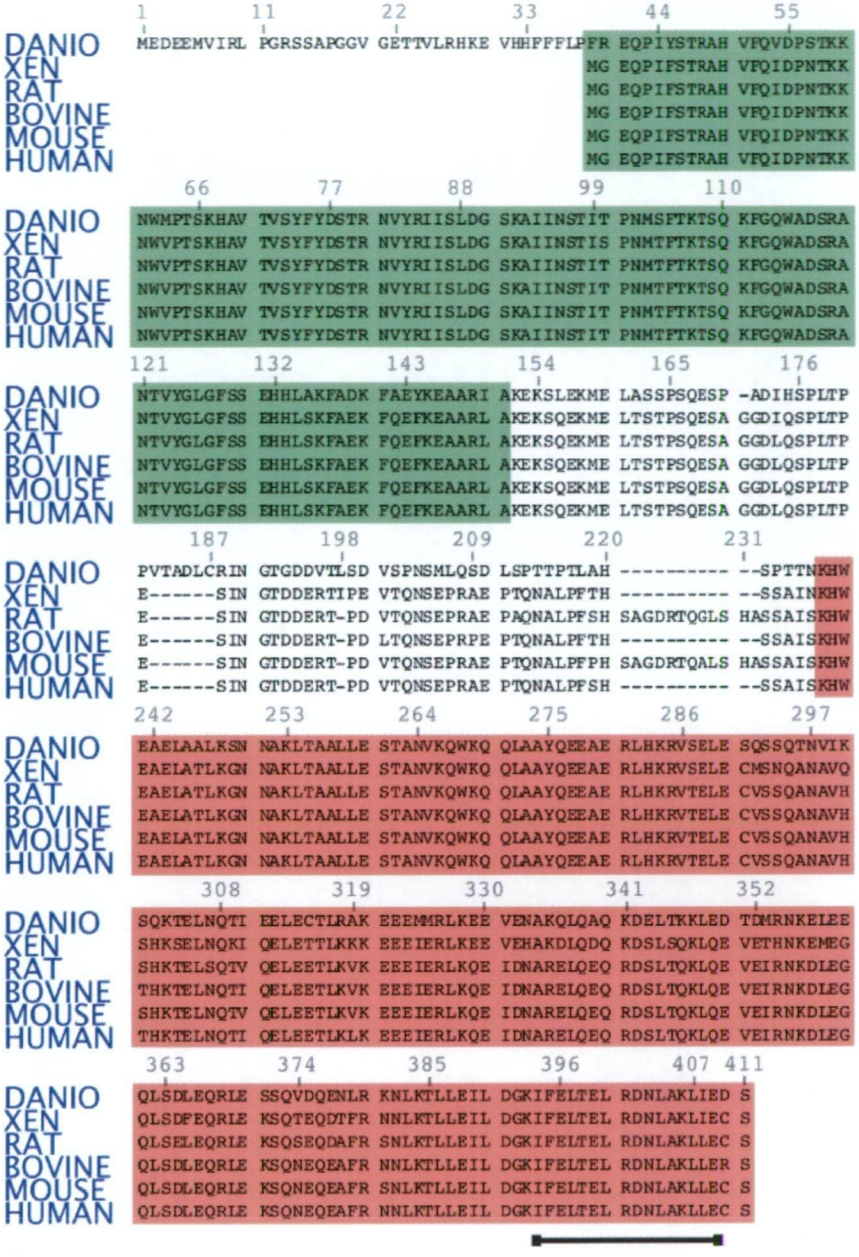


Figure 2.1

Homology of Homer proteins

Figure 2.1

Homology of Homer proteins

Amino acid sequences of long form Homer1 from human, rat, mouse, bovine, *Xenopus* and a putative zebrafish homolog showing the high degree of amino acid similarity (85%) and identity (70.6%) between species studied so far. EVH1 and coiled-coil domains are shaded green and red respectively. Polyclonal Homer 1b/c antibody epitope located at residues 395-409, is denoted by the bold line.

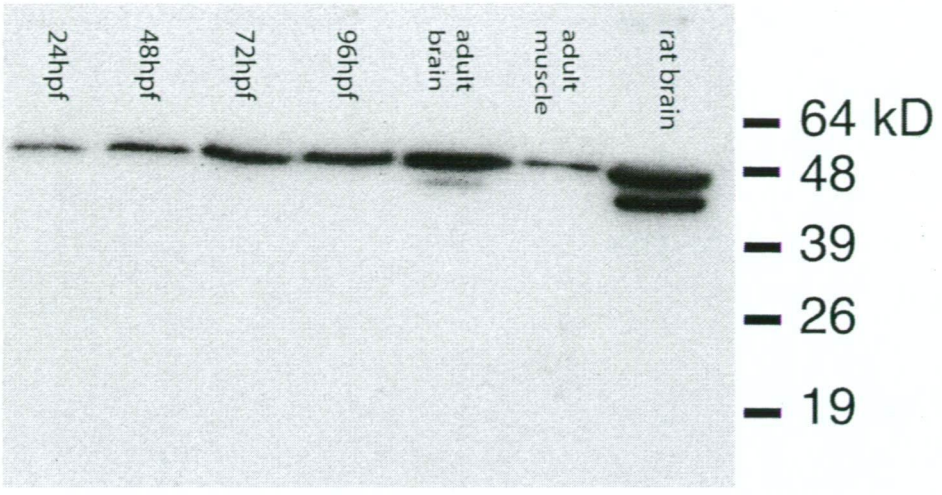


Figure 2.2

Developmental regulation of Homer in the zebrafish.

Figure 2.2

Developmental regulation of Homer in the zebrafish.

Figure showing zebrafish embryo and larval tissues immunoblotted with a polyclonal Homer 1b/c antibody. The Homer 1b/c polyclonal antibody detected a band at approximately 47kD in protein samples extracted from whole zebrafish embryos at 24, 48, 72 and 96 hpf, in addition to adult zebrafish brain and skeletal muscle. Adult rat brain (cortex) was used as a positive control. The level of Homer 1b/c expression increased gradually from 24 hpf through to adult. At high expression levels, the Homer 1b/c band appeared as a doublet (adult fish and rat cortex samples) as described previously (Xiao *et al.*, 1998). (All lanes were loaded with exactly 30 μg of protein)

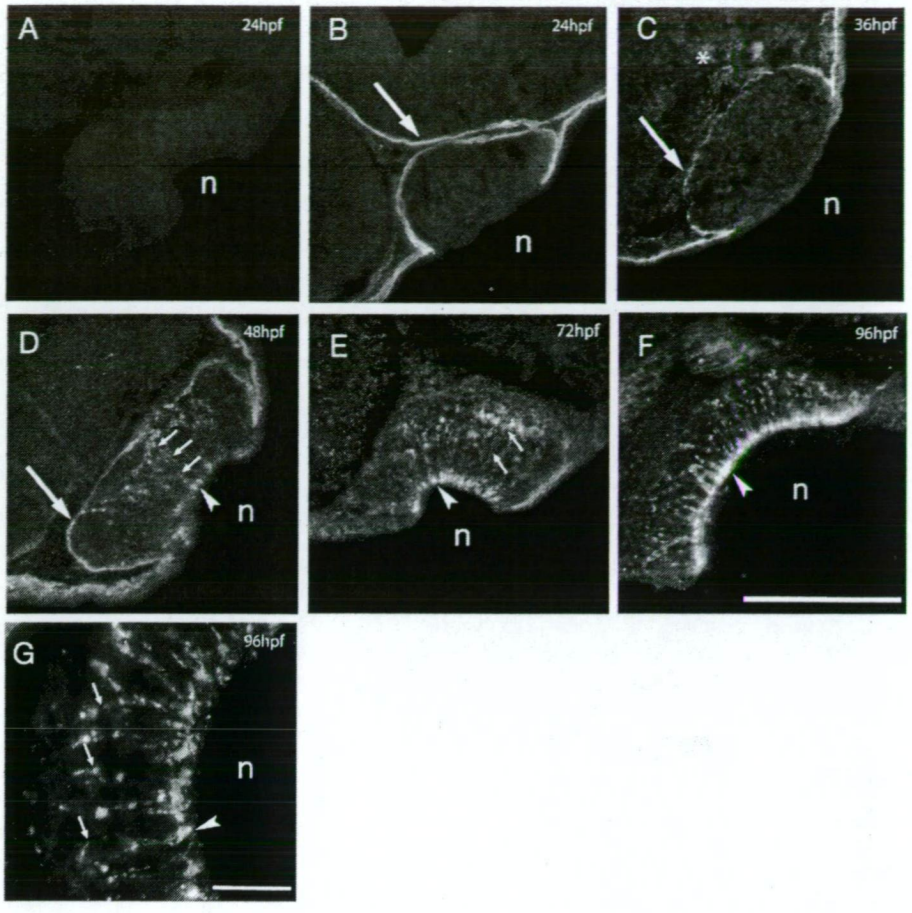


Figure 2.3

Homer expression in the developing zebrafish olfactory system.

Figure 2.3

Homer expression in the developing zebrafish olfactory system.

Homer-IR in the developing zebrafish olfactory system **A.** Horizontal section through the olfactory placode of a 24 hpf embryo treated as a no-primary negative control, demonstrates the specificity of the polyclonal Homer 1b/c antibody in zebrafish tissue. **B.** At 24 hpf, specific Homer-IR delineated the boundary of the olfactory placode. Expression was evident in the presumptive basement membrane (arrow). **C.** Homer-IR continued to be restricted to a basement membrane localization (arrow) in 36 hpf embryos. There was some evidence to suggest early Homer-IR in the developing forebrain primordium (*). **D.** At 48 hpf, Homer-IR was detected throughout the olfactory placode. Basement membrane expression was diminished, but still evident (large arrow). Homer-IR was evident along the naso-temporal axis of the placode, as large discrete puncta (small arrows) and at the apical dendritic surface (arrowhead). **E.** At 72 hpf, strong Homer-IR was evident along the apical border (arrowhead), consistent with a dendritic localization. Strong perinuclear staining was evident throughout the naso-temporal axis of the placode (small arrows). Basement membrane expression was no longer apparent. **F.** At 96 hpf, Homer-IR was most prominent in dendrites along the nasal border of the placode (arrowhead). **G.** Higher magnification illustrates Homer-IR within the OSN dendritic-knobs (arrowhead). Large Homer-IR puncta were also evident in a perinuclear pattern (small arrows). Abbreviation: n = nasal cavity. Scale bar in F is 40 μm and applies to panels A - F. Scale bar in G is 20μm.

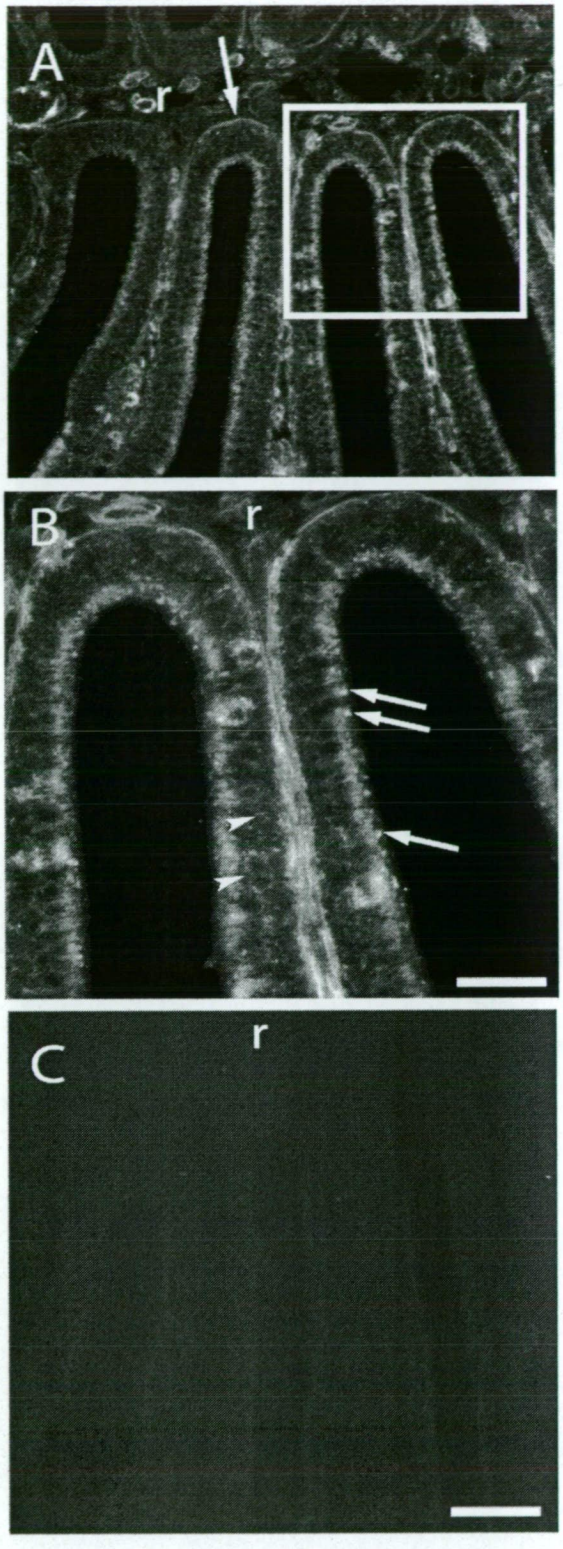


Figure 2.4

Homer in the adult olfactory sensory epithelium

Figure 2.4

Homer in the adult olfactory sensory epithelium

Horizontal sections through adult neuroepithelium immunostained for Homer-IR. **A.** Horizontal section of an olfactory rosette shows specific Homer-IR in the basement membrane of the sensory epithelium (arrow). **B.** The boxed region in A shown at higher magnification illustrates Homer-IR in OSN dendritic knobs (arrows), in addition to a punctate perisomatic pattern (arrowheads). **C.** Horizontal section through the adult olfactory rosette treated as a no-primary negative control, demonstrates the specificity of the polyclonal Homer 1b/c antibody in adult zebrafish olfactory tissue.

Abbreviation: r = midline raphe. Scale bar in C is 50μm and applies to A and C. Scale bar in B is 25μm.

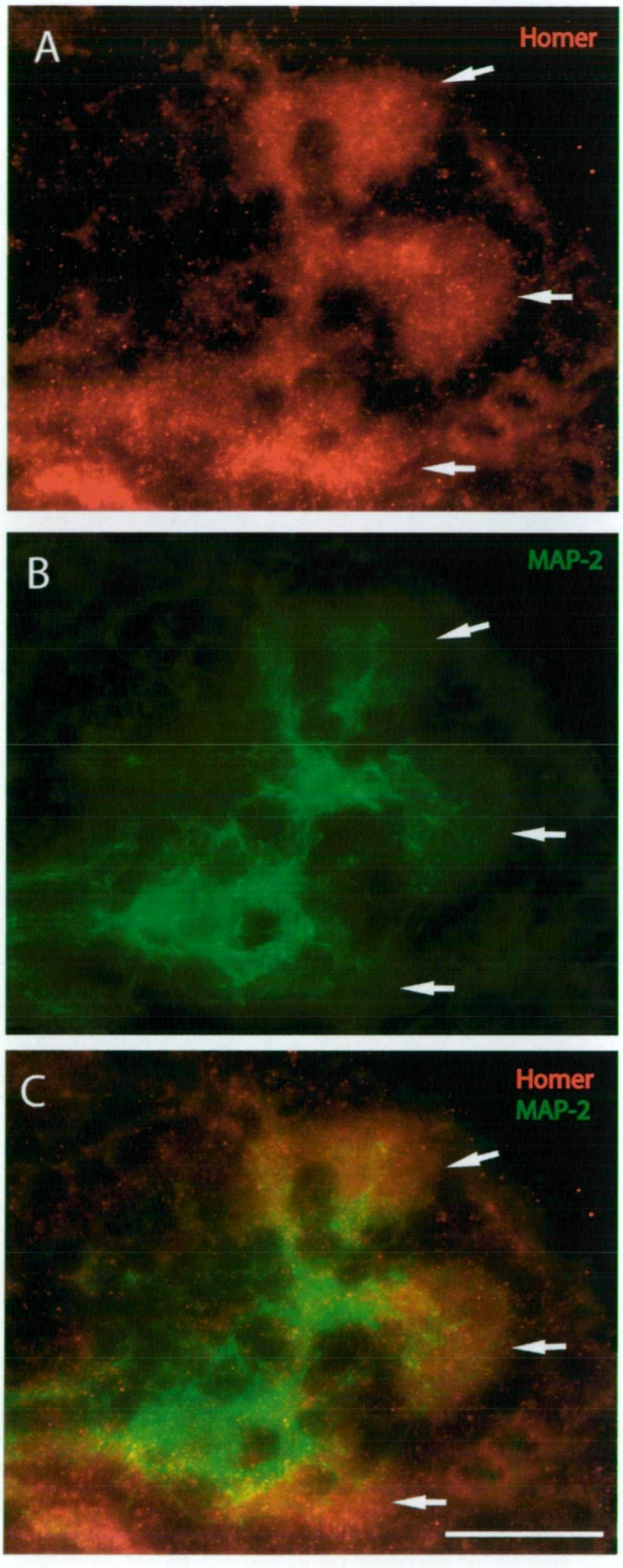


Figure 2.5

Homer and MAP2 expression in the larval forebrain

Figure 2.5

Homer and MAP2 expression in the larval forebrain

Horizontal sections of larval forebrain immunostained for Homer and the dendritic protein, MAP-2. **A**. Homer-IR was prominent in the glomeruli of the olfactory bulb (arrows). **B**. The developing neuropil and dendritic arbors of the olfactory glomeruli (arrows) are highlighted by staining with the dendritic marker, MAP-2. **C**. The merged image of A and B illustrates the close association of Homer-IR and MAP-2, particularly in the olfactory glomeruli (arrows). Anterior is to the right. Scale bar is 10μm.

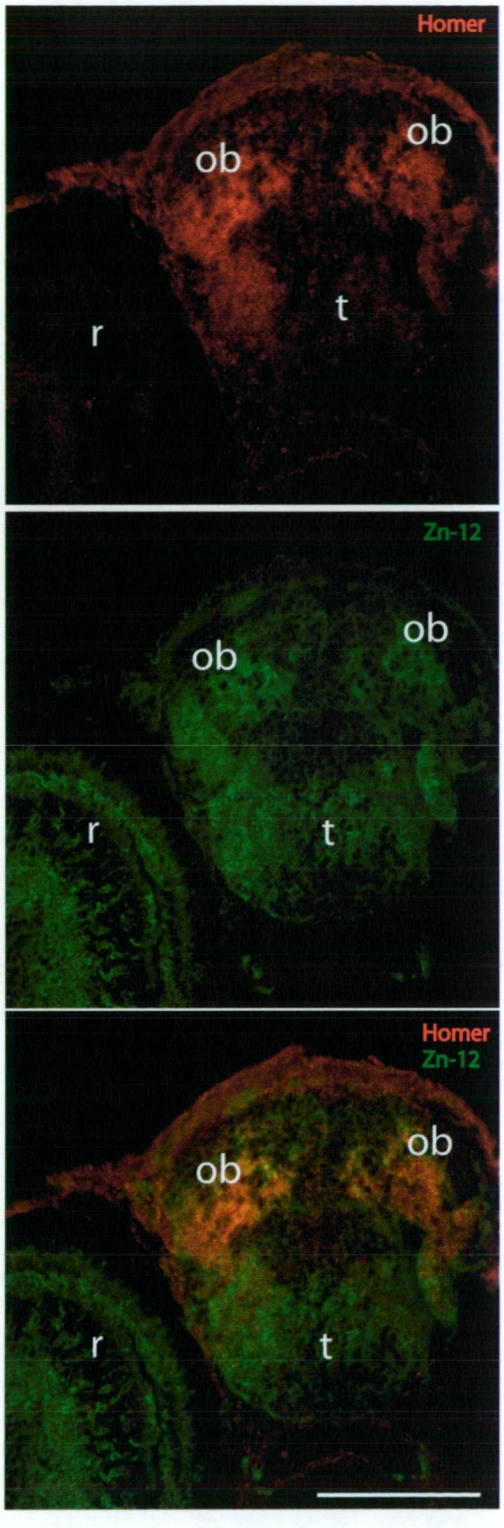


Figure 2.6

Homer in olfactory glomeruli of 96 hpf larvae

Figure 2.6

Homer in olfactory glomeruli of 96 hpf larvae

Homer and ZN-12 immunostaining in coronal sections through the 96 hpf larval forebrain. **A.** Homer-IR was concentrated in the neuropil of the olfactory bulbs. **B.** The zebrafish neural cell surface marker, ZN-12 localises to the neuropil of olfactory glomeruli as well as other areas of developing telencephalon and neuropil of the retina. **C.** The merged image of A and B illustrates the co-localisation of Homer and Zn-12, particularly within the olfactory glomeruli. Anterior is towards the top. Abbreviations: r = retina; t = telencephalon; ob = olfactory bulb. Scale bar is 100µm.

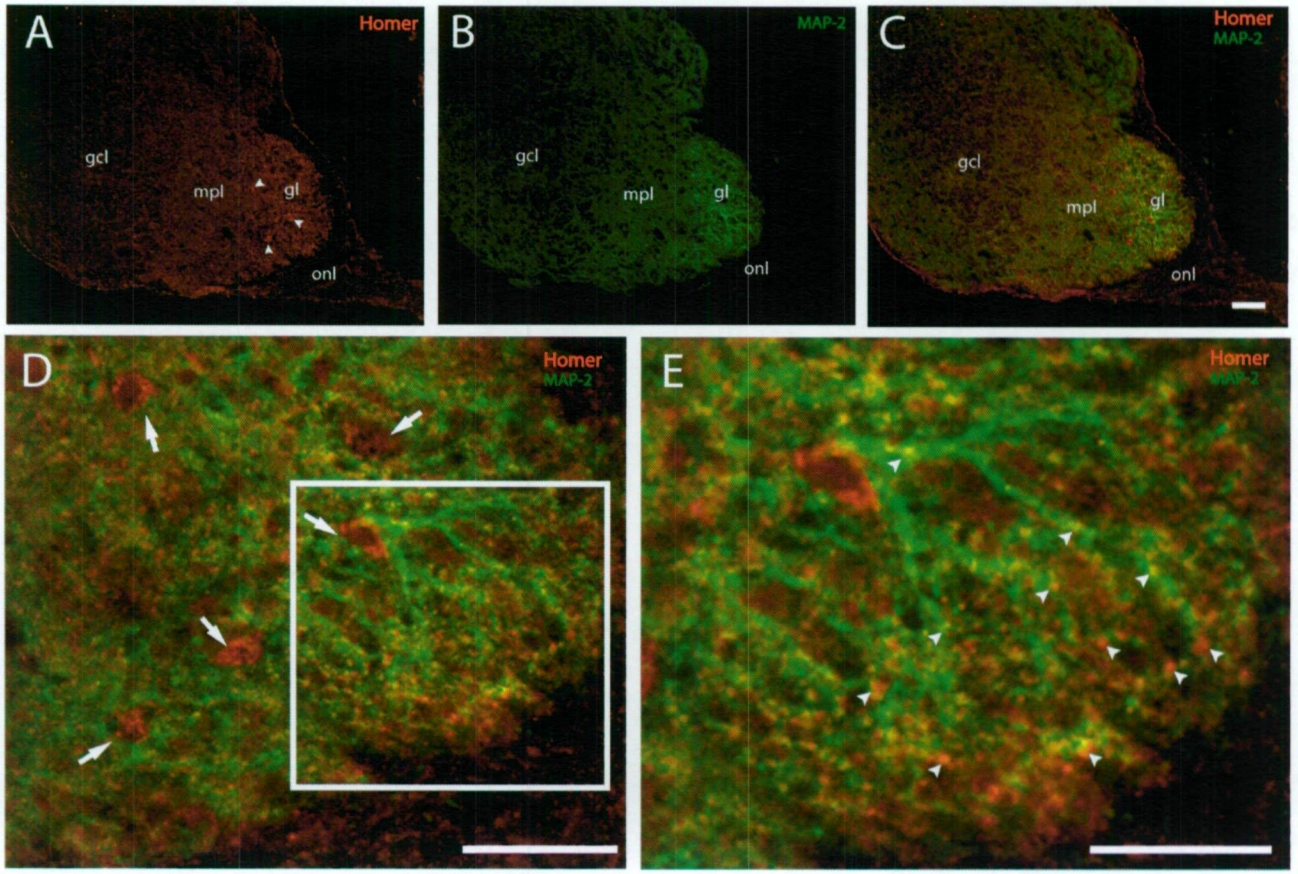


Figure 2.7 **Homer in primary circuits of the adult olfactory bulb.**

Figure 2.7

Homer in primary circuits of the adult olfactory bulb.

Figure showing parasagittal sections cut through the adult forebrain and immunostained for Homer and the dendritic marker, MAP-2.

A. Examination of the adult olfactory bulb revealed diffuse Homer-IR, especially prominent within the glomerular layer, in addition to large Homer-IR positive soma (arrowheads). **B.** MAP-2 was used to demonstrate the dendritic arbors of the glomerular layer and neuropil of the mitral cell/plexiform layer. **C.** Merged image of A and B suggests a co-localization of Homer-IR and MAP-2 particularly in the plexiform and glomerular layers. **D.** The large Homer-IR soma located along the border of the glomerular and mitral cell/plexiform layers shown at higher magnification are consistent with a mitral cell identity (arrows). **E.** The boxed area in D shown at higher magnification reveals a mitral cell dendritic arbor projecting into the glomerular layer where extensive co-localization of Homer-IR and MAP2 positive puncta is evident (arrowheads). Abbreviations: onl = olfactory nerve layer; gl = glomerular layer; mpl = mitral cell/plexiform layer; gcl = granule cell layer. Scale bar in C is 50μm and applies to panels A-C. Scale bar in D is 10μm. Scale bar in E is 5μm. Dorsal is towards the top of the page and anterior is to the right.

2.4 Discussion

These experiments show the expression of the cytosolic scaffolding protein, Homer, during zebrafish olfactory system development. Homer expression was found to be developmentally regulated with maximal levels of protein apparent by 72-96 hpf, concomitant with important behavioural interactions of zebrafish larvae with the environment (Westerfield, 1995). Homer expression continued through to adult life and was present in functionally relevant areas of the adult olfactory apparatus and midbrain, supporting the hypothesis that Homer expression is required for functional circuit development.

Homer is highly conserved across species including zebrafish where its ontogeny was characterised with a polyclonal antibody directed against the 18 C-terminal residues of rat Homer 1b/c. This epitope is located in an extremely well conserved area of the Homer protein coiled-coil domain, justifying its use in the current experiments. Homer-IR was first detected at 24 hpf in the basal layers of the developing placode along the presumptive basement membrane. Indeed, Homer expression appears to define the boundary of the placode at a time when early pioneer neurons are projecting axons into the developing olfactory forebrain (Whitlock and Westerfield, 1998). The trajectories of pioneer axons are highly stereotypic, clearly responding to very precise guidance cues (Whitlock and Westerfield, 1998). The presence of a protein such as Homer with known receptor clustering functions (Ango *et al.*, 2000; Ango *et al.*, 2002; Roche *et al.*, 1999; Usui *et al.*, 2003) at the cell boundary of the placode suggests that it could contribute to specific placode-sensitive pathfinding cues. An explanation for such an observation would warrant further, more detailed subcellular analysis, however a basement membrane or cell boundary localisation for Homer has been demonstrated *in vitro* (Shiraishi

et al., 1999). It has already been shown in the developing *Xenopus* optic tectum that appropriate and contextual Homer expression is crucial for correct pathfinding (Foa *et al.*, 2001). While this expression pattern is striking in its appearance and possible implications for SON pathfinding out of the developing placode, it requires validation since Homer has a cytosolic distribution. Control experiments using morphant embryos would be instructive in this regard.

Homer-IR was detected within the cellular milieu of the placode at 48 hpf as discrete puncta along the boundaries of epithelial cells. While such a distribution may simply represent cytosolic or perisomatic localization, it may also represent cell-cell junctions. Interestingly, this Homer expression pattern paralleled the ontogeny of the gap junctional protein, connexin, in the zebrafish lens, where 36-48 hpf is a critical period for expression (Cheng *et al.*, 2003) and in the olfactory system where 48 hpf was a critical period for the development of functional gap junctions in the developing placode (Weber and Ross, 2003). A similar cell-cell expression pattern was described in *Xenopus* (Foa *et al.*, 2005), and a cell-cell junction role has been previously described for Homer proteins (Shiraishi *et al.*, 1999).

Significant Homer expression in OSN dendrites was first detected at 48-72 hpf. Concomitantly, at this developmental time perod, larvae are just hatching. For the next 6-18 hours the newly hatched larvae lie on their sides at the bottom of their glass bowls with very little, if any, active locomotion or exploration of their environment (Westerfield, 1995). The only observable reaction to stimuli appeared to be movement of the eyes and rapid swimming escape reflexes after mechanical stimulation (data not shown). At 72-96 hpf, the zebrafish larvae become increasingly active and aware of their immediate

environment. Inflation of swim bladders, predation and active avoidance behaviours are all occurring at this time (Kimmel *et al.*, 1995). Significantly, at 72-96 hpf, there was a marked translocation of Homer to more apical or dendritic areas of the placode which persisted into the adult sensory epithelium. Such a dendritic localization is consistent with previous descriptions of Homer function, in which it acts as a scaffold protein at the post synaptic density in the mammalian cortex and hippocampus (Xiao *et al.*, 2000). Furthermore, the coincident ontogeny of Homer-IR in OSN dendrites at 48-72 hpf with early odorant receptor expression (Barth *et al.*, 1996) is consistent with the hypothesis that Homer has an organizational role in odorant receptor function. Such a role could contribute significantly to the development of complex behaviours.

The expression of Homer during development is consistent with a role in the functional wiring of the primordial and adult olfactory bulb. By 72 hpf, zebrafish larvae have developed a fully functional, albeit primitive olfactory system, with all of the cellular and neural elements required for active olfaction. At this developmental timepoint, Homer is prominent in the zebrafish forebrain, particularly within the olfactory glomeruli, where it is localized in close apposition with dendrites. In the adult bulb, Homer-IR within the glomeruli appears much more refined. There is significant expression within mitral cell bodies which significantly, appears to be localised to dendrites within the glomeruli. Future experiments utilizing electron microscopy would help to confirm the localisation of Homer to dendrites. Mitral cells relay olfactory information to higher brain centres and thus it is likely that Homer plays a role in refining and/or strengthening synaptic connections in the primary relay circuitry of the olfactory system, as it does in the rodent hippocampus (Sala *et al.*, 2005).

Propagation of olfactory stimuli is the result of a series of molecular signals originating with the binding of odorant molecules to specific receptors in the distal portion of OSN's. The odorant receptor binding and subsequent intracellular signaling cascade requires a suite of transduction molecules to be present in the neuroepithelium. These experiments show that Homer proteins are present in the olfactory neuroepithelium. Furthermore, Homer proteins are known to function in trafficking and clustering of mGluR in the mammalian nervous system (Ango *et al.*, 2000; Ango *et al.*, 2002; Ciruela *et al.*, 2000; Foa *et al.*, 2001; Roche *et al.*, 1999; Tadokoro *et al.*, 1999; Usui *et al.*, 2003; Xiao *et al.*, 1998). Homer, therefore, is well placed to mediate extracellular receptor binding to the release of intracellular signaling molecules such as calcium via IP_3 and/or RyR receptors. The ability of Homer to mobilize and orchestrate the density and spatial distribution of mGluR is highly significant since glutamate appears to be an important neurotransmitter in the zebrafish olfactory bulb (Edwards and Michel, 2002). It is also possible that Homer is required for the trafficking and/or clustering of odorant receptors themselves.

Homer protein is developmentally regulated and present in functional elements of the developing zebrafish nervous system at behaviourally significant time-points. In light of the highly stereotypic, precise nature of olfactory circuitry, it is plausible that Homer proteins function in orchestrating the molecular signals needed to initiate, support and refine these synaptic connections. To completely understand the nature of these interactions, future experiments in the zebrafish developmental model would include morpholino knock down of Homer1 in developing embryos with subsequent detection of resultant pathfinding defects. Analysis would make use of the optical transparency of these embryos to reconstruct growth cone behaviours

and axon trajectories at important choice points in axons emanating the olfactory placode. These experiments could demonstrate a necessity for Homer protein in axon guidance *in vivo* as well as a more generalised role for Homer in the trafficking and clustering of post-synaptic receptors and signalling molecules.

A more thorough examination of the cell signalling events operating within the growth cone, however, would be facilitated by an *in vitro* approach utilising pharmacology and a behavioural readout such as a growth cone turning assay. It is the latter of these two experimental approaches that will form the basis of the following chapters.

Chapter 3

Motility of dorsal root ganglion (DRG) sensory neurons in a growth cone turning assay

3.1 Introduction

The hypothesis presented in this thesis, that Homer facilitates the intracellular signalling of extracellular guidance cues, is based on the assumption that Homer functions at the growth cone to influence motile events and thereby coordinate axon guidance. Motile changes and subsequent changes in axon trajectory can be studied either *in vivo* by reconstructing developing circuits in the intact animal or *in vitro* using dissociated neurons. The ability to observe the complete repertoire of growth cone behaviours in the intact nervous system, with cellular and molecular interactions in place, is an experimental approach that has yielded many fundamental insights into early neural development, especially in non-vertebrate species. However, while using an *in vivo* approach provides valuable information, molecular interactions can be difficult to interpret due to the complexity of the embryonic cellular milieu (Bastiani *et al.*, 1984; Kuwada, 1986; Raper *et al.*, 1984; Wilson *et al.*, 1990). In addition, sophisticated imaging technologies are required to observe cells deep in embryonic tissue over long periods of time (Cline *et al.*, 1999; Knott *et al.*, 2006; Witte *et al.*, 1996).

Previous work has established the necessity for Homer in axon pathfinding *in vivo* (Foa *et al.*, 2001) and its signal transduction functions at the PSD (Kato *et al.*, 1998; Tu *et al.*, 1999; Tu *et al.*, 1998; Yuan *et al.*, 2003). It has not been established, however, if Homer has similar functions in the growth cone. An *in vitro* approach will facilitate our understanding of the intracellular signalling mechanisms mediated by Homer that operate within the growth cone. Using pharmacologic and/or genetic manipulations of intracellular signalling partners, the hypothesised role of Homer could be assessed by recording changes to predicted growth cone behaviour or motility *in vitro*. This approach allows the

analysis of complex signalling pathways and has been used recently to elucidate similar roles for BDNF and netrin-1 in growth cone motility (Li *et al.*, 2005; Wang and Poo, 2005).

Historically, *in vitro* growth cone behaviour experiments have relied on two approaches, either the observation of dissociated neurons in relatively sparse primary culture conditions over short periods of time or the explantation of neural tissue fragments over longer time courses. Explantation of neural tissue into an *in vitro* culture system allows the observation of isolated axons and growth cones while still maintaining intercellular integrity through somatic connections in a preparation that can be likened to a physiological slice preparation. These approaches have been used to examine the interactions between growth cones from differing neural tissues (Kapfhammer and Raper, 1987), or astroglia (Baird *et al.*, 1992). The chemotactic behaviour of large numbers of collectively interconnected axons from discrete areas of embryonic brain can also be observed under the influence of a asymmetrically applied guidance molecules such as semaphorins (Pozas *et al.*, 2001). This approach allows a large number of interactions and behaviours to be assessed and quantitated. These techniques have been refined by utilising "stripes" of permissive and non-permissive substrates laid down on culture plates, where the preferential adhesive behaviours of individual or fasiculating axons can be quantitated (Snow *et al.*, 1990). While these approaches have greatly enhanced our understanding of broad guidance mechanisms, the underlying changes in growth cone motility and the signalling events that precede them have yet to be elucidated.

The morphology and growth characteristics of growth cones in culture can respond to a variety of chemotactic stimuli, including serotonin (Haydon *et al.*,

1984), electrical activity (Cohan and Kater, 1986; Davenport *et al.*, 1993), or to interacting cells such as fibroblasts, sclerotome cells or glia (Polinsky *et al.*, 2000; Steketee and Tosney, 1999). Many studies have attempted to correlate motility with molecular signalling events, however the exact nature of the events remains elusive. Work by Gunderson and Barrett (1979) showed that NGF can promote chemotactic cell growth rather than merely providing a cell survival role. By quantifying the changing trajectories of chick DRG axons with varying positions of a NGF-filled micropipet in a perfused culture chamber, Gunderson and Barrett (1979) hypothesised that a chemotactic NGF gradient was produced in culture, to which the DRG were actively responsive. The power of this "growth cone turning" assay was subsequently realised in the seminal work of Lohof *et al* (1992) using *Xenopus* spinal neurons. Their method of constructing a microgradient by pulsatile ejection from a micropipette was rigorously characterised and still forms the basis of similar turning experiments (Bartoe *et al.*, 2006; Brunet *et al.*, 2005; Leung *et al.*, 2006; Tojima *et al.*, 2007).

The growth cone turning assay has been used to uncover the molecular correlates of growth cone turning in response to acetylcholine (Zheng *et al.*, 1994), glutamate (Zheng *et al.*, 1994), Netrin-1 (Ming *et al.*, 1997) and electrical stimulation (Ming *et al.*, 2001). The role of cyclic nucleotides and G-protein signalling in growth cone guidance has been consolidated with turning experiments employing the added power of pharmacological bath application of modulators of cytosolic signalling such PKA and PKC inhibitors and membrane-permeant nucleotide analogues (Nishiyama *et al.*, 2003). This study demonstrated the crucial role of cAMP as a second messenger molecule in growth cone attraction and cGMP in growth cone repulsion. Significantly, other experimental approaches have supplemented the functional responses

of the turning assay with parallel measurements using calcium imaging and electrophysiology, effectively teasing out molecular correlates of motile responses from complex downstream signalling mechanisms (Song *et al.*, 1998; Song *et al.*, 1997; Xiang *et al.*, 2002).

The hypothesis that Homer proteins facilitate the transduction of guidance cues to the cytosol can be directly addressed by using a growth cone turning assay. The assay was adapted from protocols described by Lohof *et al* (1992) and used to examine turning behaviour of embryonic rat DRG growth cones. These sensory neurons are a well described cell type from a relevant model organism. They have been used extensively in similar assays in chick and *Xenopus* (Challacombe *et al.*, 1996, 1997; Gundersen and Barrett, 1979; Kapfhammer *et al.*, 1986; Ming *et al.*, 2001; Ming *et al.*, 1997; Ming *et al.*, 2002; Raper and Kapfhammer, 1990; Song *et al.*, 1998; Zheng *et al.*, 1996). *In vivo*, DRG neurons are capable of long navigational journeys, connecting peripheral sensory receptors to the CNS. In dissociated primary culture, they display long axons with effusive, highly motile growth cones, making them ideal cells with which to study growth cone motility. Previous work has shown that Homer is developmentally regulated in sensory systems and correlates well with behavioural development (Chapter 2). This chapter describes and characterises DRG growth cone motility in chemotactic microgradients, demonstrating that DRG neurons respond appropriately to established and novel guidance cues *in vitro*.

3.2 Materials and methods

3.2.1 Cell culture

Thoracic dorsal root ganglia from embryonic day 16-18 Hooded Wistar rat were mechanically dissociated into sensory neuron medium (SNM) comprising Dulbecco's Modified Eagle Medium / Ham's F-12 medium 1:1, (SAFC Biosciences), foetal calf serum (5%v/v), penicillin G (100U/ml), streptomycin (100 μg/ml), nerve growth factor, (NGF, 50 ng/ml, Sigma-Aldrich) and N2 neural medium supplement (1:100, Gibco). Cells were plated at low density onto poly-ornithine (1 mg/ml, Sigma) and laminin (100 μg/ml, Gibco) coated glass coverslips embedded into 35 mm plastic dishes (Iwaki, Asahi, Japan). Dishes were maintained in a 5% CO_2 / 95% room air incubator at 37°C. Dishes were incubated for at least 4 hr. Only isolated growth cones from actively protruding axons were used for analysis.

3.2.2 Micropipettes

Micropipettes used to develop microgradients were pulled from borosilicate glass capillaries with internal filament. Capillary glass of various dimensions was examined, however consistent pipettes were pulled from 1.0 mm OD x 0.58 mm ID glass (with filament). Pipettes were pulled using a microprocessor-controlled puller (P-87, Sutter Instrument Co., USA) and finally fire-polished to an orifice opening of approximately 1.0-1.4 μm with a microforge (MF-830, Narashige, Japan)

3.2.3 Growth Cone Turning Assay

Turning assays were performed as previously described (Li *et al.*, 2005b; Lohof *et al.*, 1992; Wang and Poo, 2005). Briefly, a microgradient was produced by the pulsatile (1Hz) ejection of the guidance cues BDNF (10 μg/ml), netrin-1 (5μg/ml), phorbol 12-myristate 13-acetate (PMA, 10 μM), sema-3a (20 μg/ml), glutamate (1mM), metallothionein 1/11 (MT1/11, 3 mg/ml), metallothionein 3 (MT3, 3 mg/ml) or zinc sulphate (0.5 mM) from a fire-polished, modified patch micropipet (see above) connected to an electrically controlled pressure delivery system (Picospritzer, Parker-Hanniffin Corp., USA). Concentrations of guidance cue molecules at the growth cone have been estimated to be 10^{-3} of micropipette concentrations (Lohof *et al.*, 1992) and for the purposes of this assay were assumed to be similar. Isolated growth cones were imaged in SNM using customised phase contrast time-lapse microscopy. Multiple, averaged images were acquired every 7 sec for 30 min using custom acquisition and data analysis software (MatLab, MathWorks). Turning angles, axon extensions and greyscale intensities were measured using ImageJ (NIH). Only growth cones extending at least 10 μm in 30 min were used for analysis. Turning angles were defined as the change in axon trajectories of the distal 10 μm of axons compared to the initial starting trajectories. Attraction and repulsion were designated positive and negative angles respectively. Micropipette tips were positioned 75-85 μm from growth cones, as close as possible to the culture substrata, at a 45° angle to initial axon trajectories. Statistical analysis of turning angles (Mann-Whitney U-test) were performed using Prism 4 (GraphPad Software).

3.3 Results

3.3.1 Pulsatile ejection produces reproducible gradients

This work attempted to reproduce the earlier work by Lohof *et al* (1992) by constructing a microgradient of guidance molecules in an *in vitro* cell culture system. Micropipettes of varying profiles were examined, with the final choice of shape being the one that gave the most reproducible and easily manufactured forged tip opening while also having adequate physical strength to permit the temporary contact of the pipette tip on the base of the culture dish (Figs 3.1 A&B). Use of a microprocessor controlled pipette puller produced consistently shaped pipettes which were subsequently fire-polished.

Preliminary studies examining the shape of ejected plume from pipettes utilised the dye, Trypan Blue, in an aqueous phosphate-buffer. Using previously published ejection parameters of 10 msec pulses and 5 psi pressure (Lohof *et al.*, 1992), a circular bolus of dye was ejected and was observed to rapidly disperse into the surrounding medium (Fig 3.2). In order to determine the precision of the ejections, 8-bit greyscale images were captured at 140 msec intervals and a circular area, 25 μm in radius approximating the visible dye bolus, was quantitated. Average pixel intensities in the circular area were used as an approximation of dye concentration. Analysis of the average pixel intensities showed slight variability in the maximal dye concentration of each repetitively ejected bolus. However the area of each response over time appeared consistent, suggesting the total mass of dye ejected was similar (Fig 3.3). The variation in maximal concentrations may have been artefactual due to the image acquisition timing not being precisely phase-locked to the ejection pulses.

To more fully explore the characteristics of microgradients, especially at the outer margins where growth cones would be predicted to encounter guidance cue molecules, the gradient profile of the plume was assessed as a line scan, originating radially from the pipette tip to the visible plume margins, rather than the average pixel intensity of the visible dye bolus area shown in Figures 3.2 & 3.3. The line scan approach would be predicted to more closely approximate the concentration profile, or gradient of dye concentration (Fig 3.4). Analysis of the dye concentration close to the tip (<10 μm) show a linear decay profile over time but a more non-linear decay profile at greater (>25 μm) distances from the tip (Fig 3.5). These observations are in good agreement with work previously done using fluorescent dextrans, confirming the microgradient profile of the proposed assay (Lohof *et al,* 1992).

3.3.2 Motile responses of wild-type DRG neurons in a turning assay

Embryonic rat sensory neurons from wild-type thoracic DRG were prepared as described. After plating on laminin coated glass coverslips, cells extended lengthy axons with effusive growth cones exhibiting highly motile filopodial and lamellipodial behaviours within the 30 min assay period (Fig 3.6). Resolution of many of the fine filopodia was impossible due to optical and image acquisition limitations. There was considerable heterogeneity in the responses as a significant number of cells either did not extend sufficient distance in the 30 min assay timeframe or conversely, exhibited complete growth cone collapse and axon retraction (data not shown).

DRG growth cones showed robust chemo-attraction towards BDNF, netrin-1 and glutamate, when compared to a control gradient of SNM, and robust

chemo-repulsion in gradients of sema-3a and PMA (Figs 3.7 A,B&C). Graphical representations of growth cone responses in this turning assay were represented either as scatter plots of final extension and trajectories (Fig 3.7B) or as histograms showing mean angles and extensions (Fig 3.7C). Turning angles were calculated exactly 30 min after initiation of micrograident. Axon extension rates were not significantly different between treatments, indicating that the general cellular health of DRG's was not compromised during the assay, even though the imaging configuration was sub-optimal with respect to the maintenance of CO_2 atmosphere while imaging was in progress. Both average turning angles and axon extension rates were similar to responses seen in other cells such as rat cerebellar granule cells and *Xenopus* spinal neurons (Li *et al.*, 2000; Song *et al.*, 1998; Song *et al.*, 1997; Wang and Poo, 2005).

3.3.3 DRG neuron responses to metallothionein I/II (MT I/II)

Turning assays allow the rapid identification of chemo-attractive or chemo-repulsive molecules. MTI/II, a putative chemo-attractant molecule, has as yet uncharacterised proliferative and chemotropic effects in a variety of experimental systems (Chung *et al.*, 2003). In a novel approach to the study of this family of molecules, physiologically relevant levels of MTI/II were tested in the growth cone turning assay and were found to elicit a robust chemo-attractive response when compared to vehicle-only micrograidents (Fig 3.8). Axon extension rates were not significantly different to control values (Fig 3.8). Micrograidents of zinc, a crucial ion sequestered by MTI/II, did not show any significant attraction or repulsion, suggesting that MTI/II's observed chemotropic role *in vitro*, was not due to release of extracellular zinc. Significantly though, axon extension rates for zinc micrograidents were

significantly lower than control or MT I/II extension rates suggesting a potential role for MTI/II in facilitating neurite motility in areas of high extracellular zinc concentration. Another related member of the MT family, MT3 has previously been shown not to have any neurite promoting effects *in vivo*. Similarly in this assay, albeit with a small number of trials, MT3 was not chemo-attractive, suggesting that the growth promoting effects seen with MTI/II rely in part, on attraction of neurites to sources of MTI/II. These data confirm the applicability of the assay in the detection and partial elucidation of novel guidance cues.

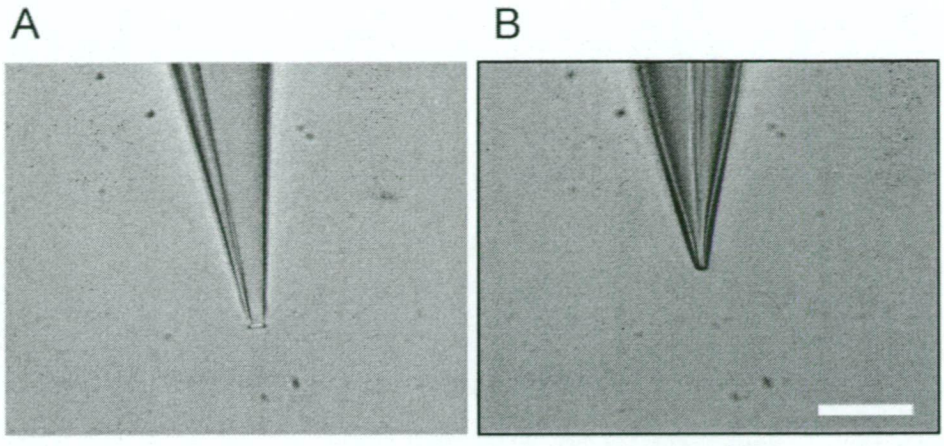


Figure 3.1

Manufacture of pipettes used in microgradient preparation

Figure 3.1

Manufacture of pipettes used in microgradient preparation

Figure showing dimensions and tip opening profiles of typical micropipettes manufactured for the growth cone turning assay. **A.** Bright field image representing a typical micropipette pulled with the microprocessor controlled puller. **B.** shows the same pipette after the tip had been fire-polished and shaped in a microforge. Scale bar is 10 μm

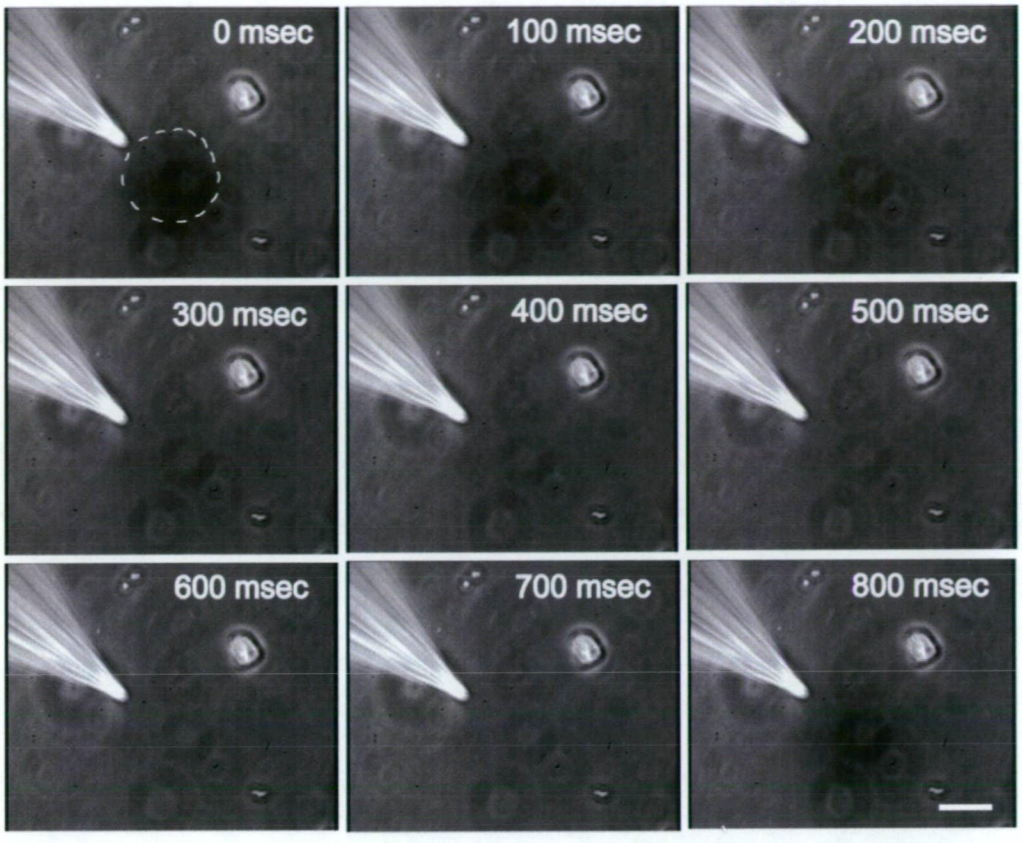


Figure 3.2

Pulsatile ejection of dye with a micropipette *in vitro*.

Figure 3.2

Pulsatile ejection of dye with a micropipette *in vitro*.

Figure showing a sequence of images taken at 100 msec intervals and demonstrating the decay of a dye plume concentration after pulsatile ejection from a micropipette. Pulses occurred at 0 msec and 800 msec. Decay of dye plume concentration is visually apparent by 400 msec after ejection. Dotted line represents circular area used to quantify dye concentration. Scale bar is 10 μm

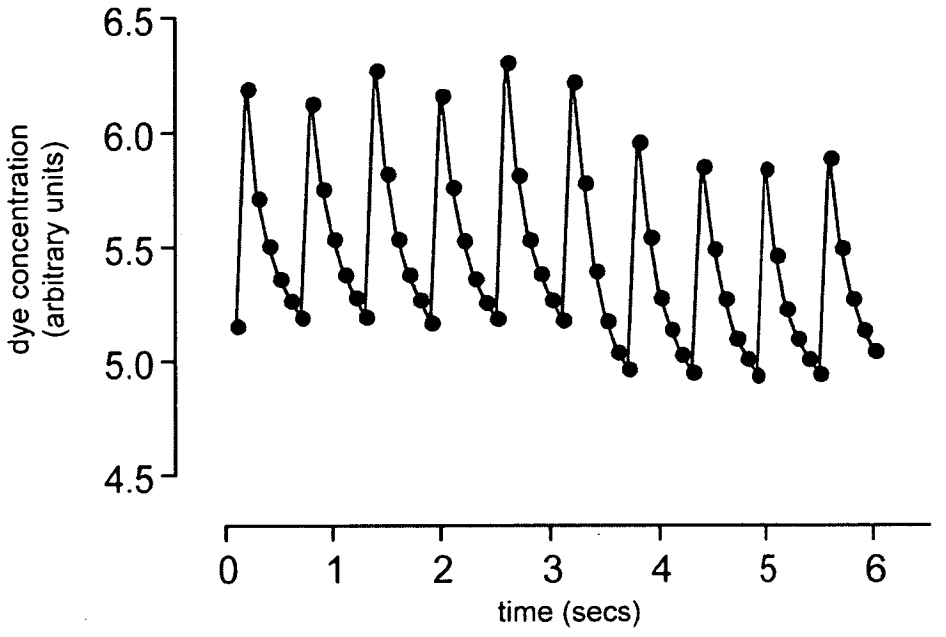


Figure 3.3

Quantitation of dye bolus concentration.

Figure 3.3

Quantitation of dye bolus concentration.

Figure demonstrating the variation in dye concentration in multiple, repetitive dye injection. The circular area defined in Fig 3.2 was used as an measure of the visible dye bolus and its mean pixel value was calculated following 10 successive pulsatile ejections. Time interval is 140 msec.

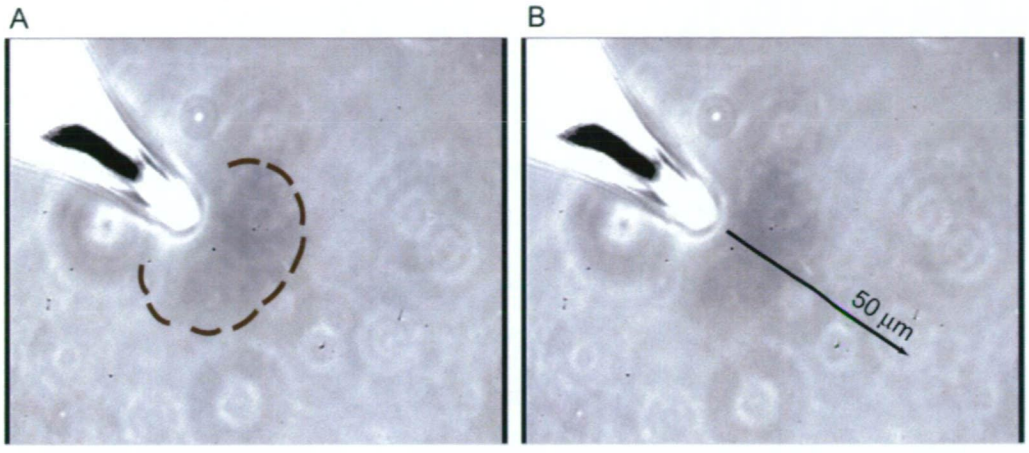


Figure 3.4

Analytical approaches used to characterise dye gradients.

Figure 3.4

Analytical approaches used to characterise dye gradients.

Figure illustrating the two analytical methods used to estimate the bolus dye concentration and the gradient concentration profile. **A.** This approach quantitated the average pixel intensity in a circular area representing the visible dye bolus. **B**. To more fully characterise the concentration gradient of dye emanating from the plume, a line scan of pixel intensities defined as a radial from the tip out to a distance of 50μm was calculated.

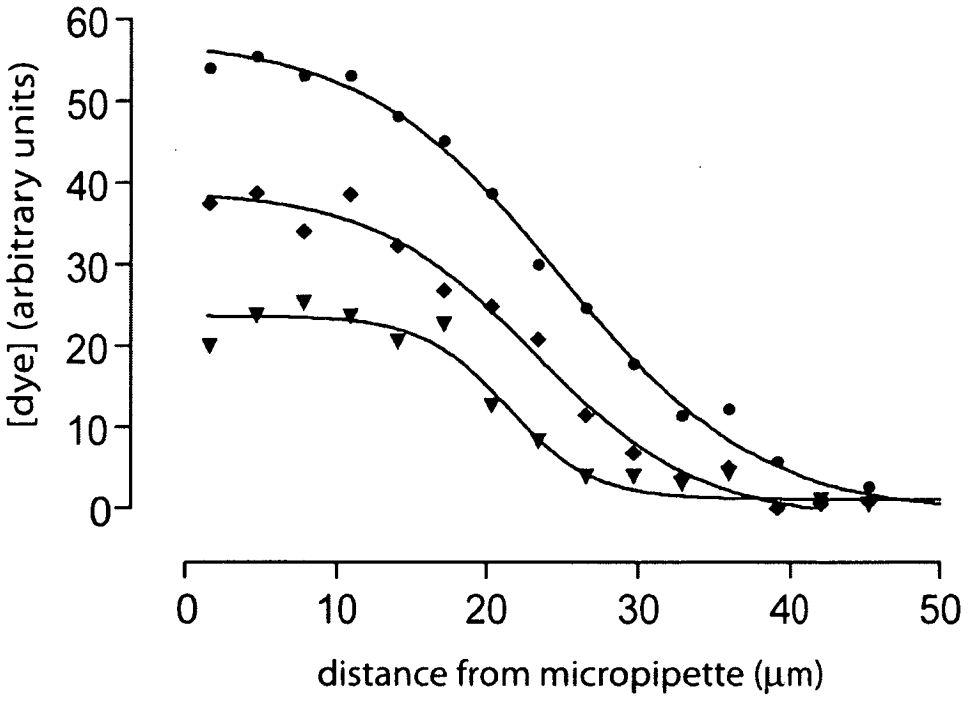


Figure 3.5

Microgradient concentration profile.

Figure 3.5

Microgradient concentration profile.

Figure shows an estimation of dye concentration as a function of distance from micropipette tip at 0, (●), 100 (◆) and 200 msec (▾). Decay of concentration close to the pipette proceeds in a linear manner. That is, concentration at 100 msec and 10 μm from tip has decayed to 50% of 0 msec value at the same position from tip. Linearity of decay decreased with increasing distance from pipette tip.

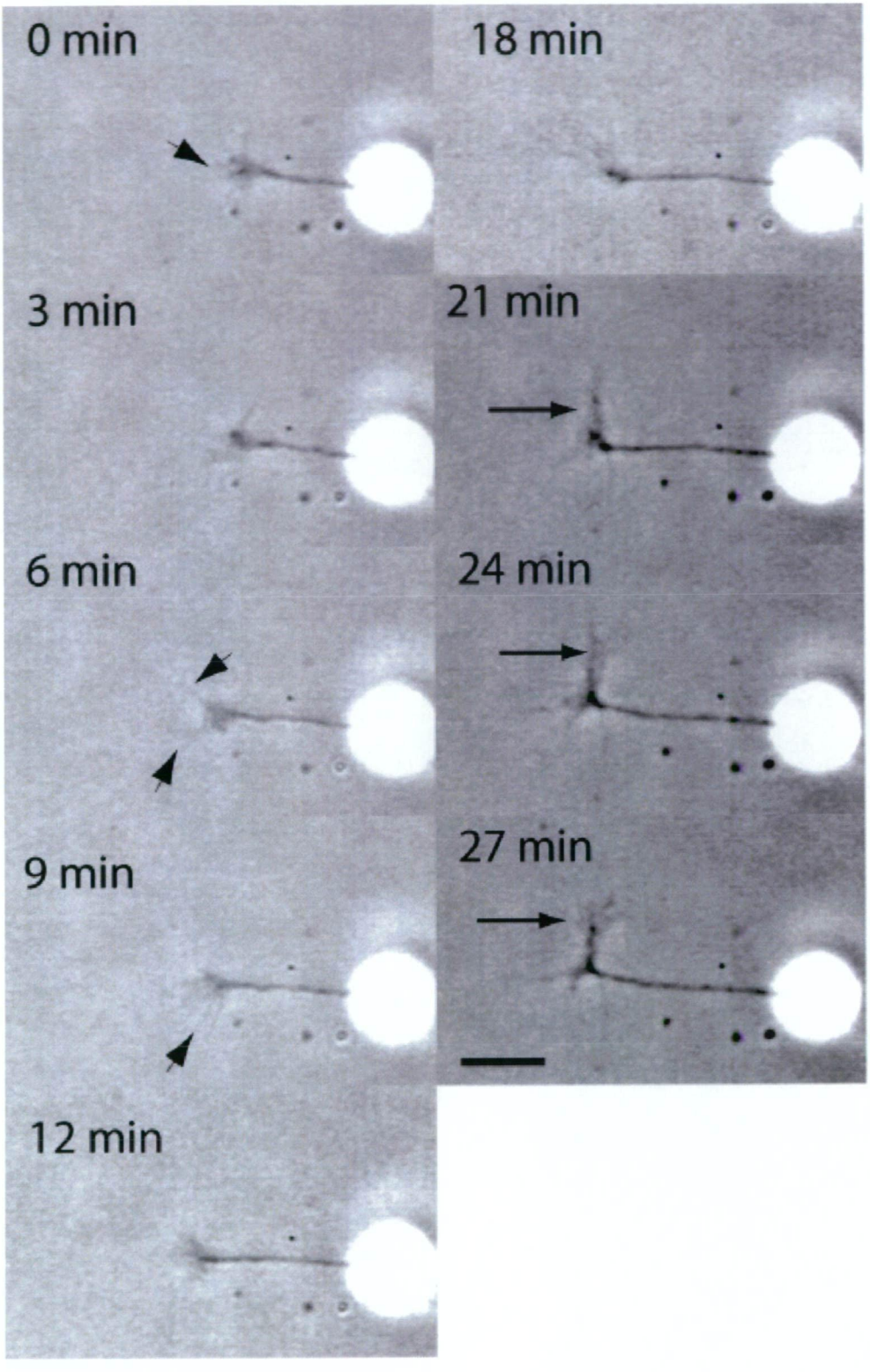


Figure 3.6

Gross morphological changes of growth cones during turning assay.

Figure 3.6

Gross morphological changes of growth cones during turning assay.

Sensory neurons from embryonic rat DRG display highly motile filopodial and lamellipodial behaviours. Time-lapse video-microscopic images taken at 3 min intervals show a variety of growth cone morphologies and behaviours eg. highly motile filopodia (arrowheads) and lamellipodial protrusion (arrows) preceding axonal branching apparent at 27min. Scale bar is 10 μm.

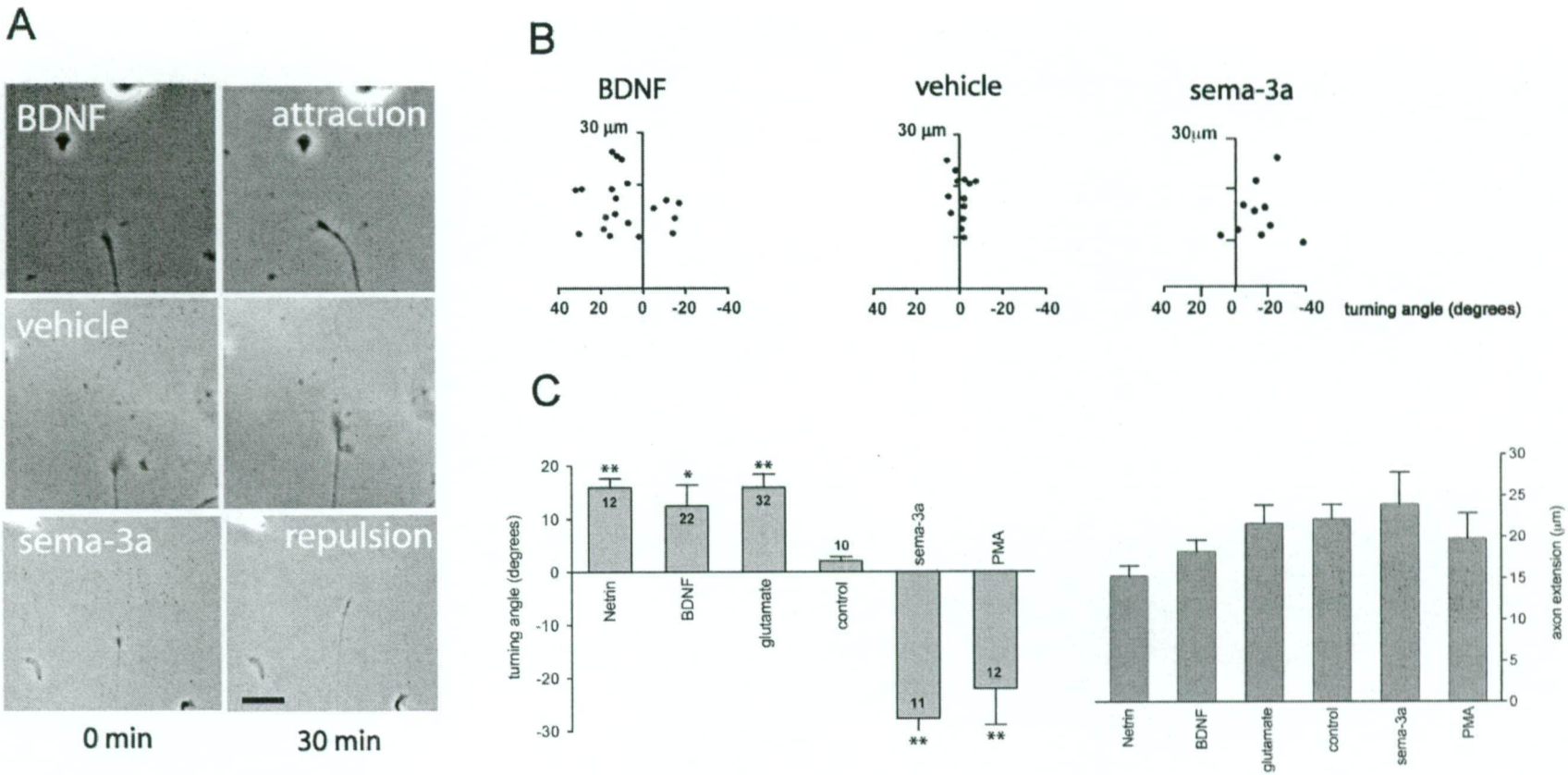


Figure 3.7 **Motile responses of DRG sensory neurons in the turning assay**

Figure 3.7

Motile responses of DRG sensory neurons in the turning assay

Motile turning responses of wild-type DRG neurons to guidance cues (concentrations of ligands in pipette) netrin-1 (5μg/ml), BDNF (10μg/ml), glutamate (1mM), sema-3a (20μg/ml), PMA (10μM) and vehicle (SNM) microgradients in the growth cone turning assay. BDNF, netrin and glutamate caused attraction while sema-3A and PMA induced repulsion of DRG growth cones. Vehicle control gradient induced random turning responses. Positive angles represent attraction and negative angles represent repulsion. **A.** Phase contrast images at the initiation of microgradient (0 min) and at the termination of the assay (30 min). Pipette containing guidance cue is at upper left quadrant in all images and out of field of view. **B.** Scatterplot depicting final turning angle (x-axis) and protrusion distance (y-axis) thereby indirectly representing variability in final growth cone trajectories for all experimental trials. **C.** Histograms depicting DRG neuron turning (y-axis) and extension responses (mean, SEM and number of observations, n). Axon extension rates did not differ significantly between any of the guidance molecules tested after 30 min. Significant differences from control values are marked as: * $p < 0.05$; ** $p < 0.005$; Mann-Whitney U-test. Error bars indicate SEM. Scale bar for (A) is 10 μm.

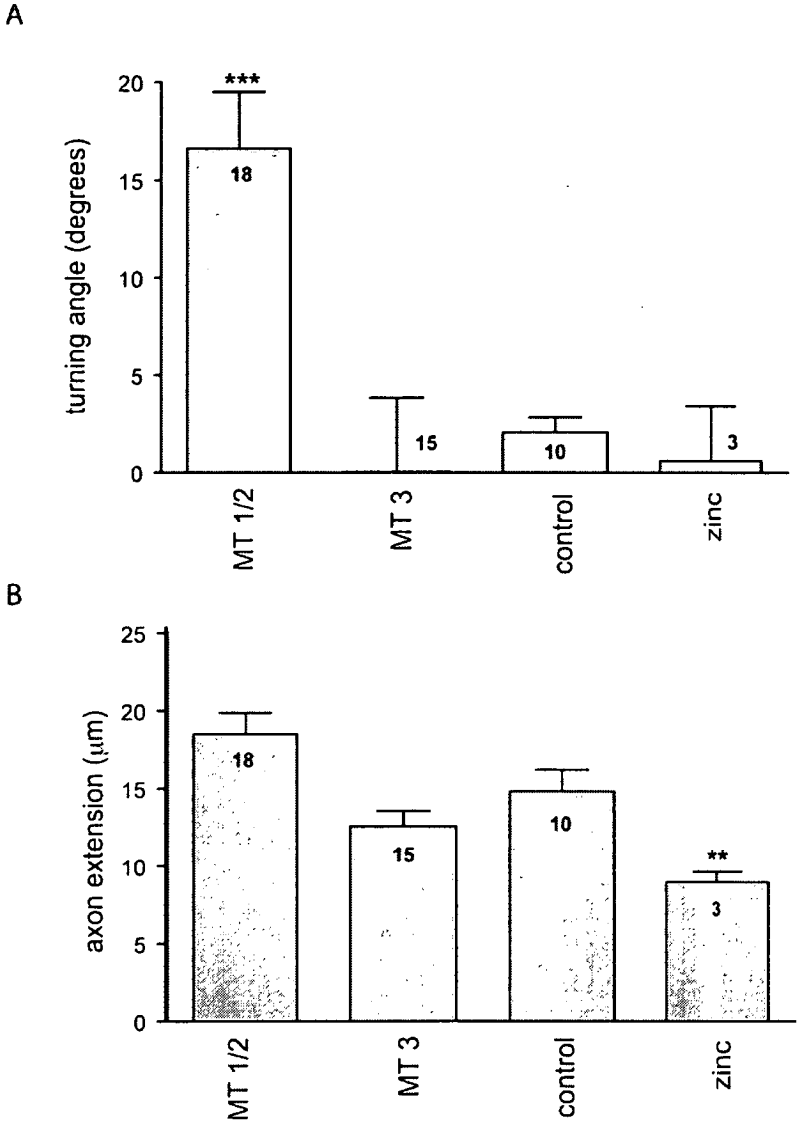


Figure 3.8

DRG growth cone responses to a microgradient of metallothioneins and zinc

Figure 3.8

DRG growth cone responses to a microgradient of metallothioneins and zinc

Average turning angles and axon extension rates of DRG in response metallothionein I/II (3mg/ml), metallothionein 3 (3mg/ml) and zinc (0.5mM) alone. Positive angles represent attraction; negative angles represent repulsion. Axon extension rates for zinc differed significantly when compared to all other treatments. Significant differences from control values are marked as: **$p<0.005$, ***$p<0.0005$; Mann-Whitney U-test. Error bars indicate SEM.

3.4 Discussion

Using experimental parameters from a well-described protocol, an *in vitro* growth cone turning assay was characterised and used to establish the motile behaviour of DRG sensory neurons. Analysis of the results indicate that the assay performed in a predictable and consistent manner. *In vitro* growth cone turning assays have been used extensively to explore the molecular mechanisms underpinning axon guidance. This technique provides considerable scope and flexibility of experimental design and can be thought of as a medium-throughput screening assay for the characterisation of putative guidance molecules. Significantly, with the incorporation of pharmacologic bath application, they provide ample scope to uncover crucial mechanistic aspects of putative guidance molecules. The assay is, however, contingent on the production of stable and reproducible microgradients of guidance molecules *in vitro*, providing statistical power to observed behaviours.

Pioneering work by Lohof *et al* (1992) characterised both the physical parameters of gradient formation and the value of the assay in understanding underlying molecular mechanisms required for growth cone motility. The protocols described in this work produced *in vitro* microgradients that are consistent with the work of Lohof *et al* (1992). With repetitive pulsatile ejection of guidance cues from micropipettes, stable gradients were formed after 3-4 min at distances of 100 μm from pipette tips.

Isolated sensory neurons from DRG have been widely studied and are relevant cells for the study of axon guidance *in vitro*. Previous studies have

demonstrated the presence of the neurotrophin receptors, TrKA and TrKB (Kaplan *et al.*, 1991; Salio *et al.*, 2005; Tuttle and O'Leary, 1998), neuropilin-1 and plexin (Fournier *et al.*, 2000) and DCC (Watanabe *et al.*, 2006) in rat DRG growth cones. The motile responses of rat DRG growth cones to the guidance cues netrin, BDNF and sema-3a do not differ significantly from other studies using chick DRG neurons and/or *Xenopus* spinal neurons in similar turning assays and *in vivo* experiments (Luo *et al.*, 1993; Masuda *et al.*, 2003; Ming *et al.*, 2001; Ming *et al.*, 1997; Serafini *et al.*, 1994; Shim *et al.*, 2005; Wang and Poo, 2005; Watanabe *et al.*, 2006; Wu *et al.*, 2006), confirming the relevance of the observed behaviours of DRG neurons and the applicability of the assay to examining the role of Homer in growth cone motility.

A novel finding arising from this study was that DRG neurons displayed a robust attraction to a putative molecular guidance molecule, MTI/II. The MT family of proteins are ubiquitous metal-binding molecules found in most body tissues and significantly, MTI/II is secreted by glial cells following CNS injury (Blaauwgeers *et al.*, 1996; Chung *et al.*, 2003; Hamer, 1986). MTI/II has been shown to induce neurite elongation and repair in both cortical axons *in vitro* and cortical wound healing *in vivo* (Chung *et al.*, 2003), suggesting that MT I/II can, under certain conditions, be chemo-attractive. The mechanism of this chemotropic function for MT I/II is unknown and further experiments with MT I/II in the growth cone turning assay would likely aid in elucidating this mechanism. In addition, metal-binding proteins and cytosolic zinc in particular, have been demonstrated in diverse mechanisms such as synaptic refinement during metamorphosis in Manduca (Rössler *et al.*, 2000) and in dysregulation of hippocampal mossy fibre sprouting in neonatal bornea disease (Williams *et al.*, 2006). The observation that DRG neurons are attracted to gradients of MTI/II, but not to free zinc, suggests that the MT effect is not mediated by the

zinc that is bound to the MT protein. Gradients of free zinc, however, significantly reduced axon extension rates compared to MTI/II, suggesting that binding zinc to MTI/II is a possible mechanism for MT-associated neurite outgrowth after CNS injury.

Further work using the now established growth cone turning assay and pharmacological interventions could investigate the mechanisms of metallothioneins. To date, there is no demonstrated mechanism to suggest intracellular signalling partners or receptors for these novel guidance molecules. It is clear that astrocytes use metallothioneins as molecules to communicate and signal tissue damage or stress. These molecules freely circulate in the tissue environment and signal to other glia and neurons. No mechanism has been suggested to explain the entry of metallothioneins into neurons. Clearly, the ability to construct a microgradient of such molecules and test whether pharmacological perturbations of endocytic pathways abolish the effects of methallothioneins would lead to a clearer understanding of neuron-metallothionein interactions.

In addition to the powerful quantitative nature of the assay, morphological changes occurring in growth cones can be quantified due to the continuous microscopic observations during the assay. As growth cones navigate through the embryonic environment they encounter diffusible cues of varying concentrations. In order to appropriately respond to these cues the growth cone must be able to effectively attenuate or adapt its motile responses to the continuously varying concentrations of guidance molecules. Recent work by (Ming *et al.*, 2002) has uncovered a mechanism involving finely balanced periods of adaptation and re-sensitisation to guidance molecules in *Xenopus* spinal neuron growth cones. In a protein synthesis- and calcium-dependent

mechanism involving MAPK, growth cones are sequentially attracted to, then repulsed from guidance cues leading to an observable "zig-zagging" trajectory. This motile behaviour was observed in a small number of cells during the course of the current study but quantitative data describing this behaviour was not collected.

More recently, it has been suggested that adaptation is not necessary to explain this behaviour and that spatial and temporal averaging of the molecular events downstream of receptor binding are sufficient (Xu *et al.*, 2005). This debate illustrates the possible limitations of interpolating motile information from turning assays into *in vivo* mechanisms, where multiple signalling gradients, in addition to contact-mediated sources of guidance, are at play to guide axons to their eventual synaptic partners. The power of *in vitro* behavioural assays, however, lies in their ability to provide specific information about signals, mechanisms or pathways that directly alter a growth cone's ability to make directional choices. This is particularly relevant when asking the question; does Homer function regulate growth cone motility?

Chapter 4

Homer expression alters the operational state of a CaMKII-CaN molecular switch

4.1 Introduction

The expression pattern of Homer in the zebrafish embryo suggests that Homer is important in developing sensory structures (Chapter 2). Significantly, Homer expression was prominent at important developmental and behavioural milestones in free-swimming zebrafish larvae, suggesting a possible role for Homer in development of sensory circuitry which is refined in these late larval stages. Furthermore, previous studies in *Xenopus* have shown the necessity for constitutive levels of Homer 1b/c for pathfinding *in vivo* (Foa *et al.*, 2001). Over-expression of Homer1c & 1a, both of which perturb constitutive Homer 1b/c levels, resulted in pathfinding and target recognition errors *in vivo.* An *in vitro* experimental approach that manipulates constitutive expression of Homer 1b/c in growth cones, while assessing their motile responses to guidance cues would be a powerful tool with which to tease out Homer function at the growth cone.

Homer function in the PSD is characterised by the orchestration of crucial calcium signalling components (for recent reviews see Mikoshiba, 2007; Worley *et al.*, 2007). $[Ca^{++}]_i$ is a crucial signalling molecule regulating growth cone motility and axon pathfinding (Ghosh and Greenberg, 1995; Kater and Mills, 1991). Growth cone responses to BDNF and netrin-1 are calcium dependent, while sema-3a responses are calcium independent (Hong *et al.*, 2000; Ming *et al.*, 1997; Paves and Saarma, 1997; Shieh and Ghosh, 1999). The spatio-temporal changes in growth cone $[Ca^{++}]_i$ following BDNF-TrkB signalling, for example, mediate the activation of CaMKII and CaN in a molecular switch-like mechanism that controls the direction of calcium-dependent growth cone turning *in vitro* (Wen *et al.*, 2004). Large changes in $[Ca^{++}]_i$ preferentially activate CaMKII to induce attraction while shallower

gradients signal through CaN to induce repulsion. These responses are further modified with changes in the basal, or resting, level of $[Ca^{++}]_i$. Low $[Ca^{++}]_i$ predisposes the growth cone to CaN activation, while normal resting $[Ca^{++}]_i$ mediates attractive calcium dependent signalling (Wen *et al.*, 2004). Another transduction mechanism acting as a molecular switch involves the cyclic nucleotide second messenger system (cAMP and cGMP). Studies focussing on calcium dependent netrin-1 signalling have shown that high cAMP levels are the molecular correlate of attraction while cGMP mediates growth cone repulsion (Nishiyama *et al.*, 2003). These two switches are not mutually exclusive, with the cyclic nucleotide molecular switch suggested to act as a negative regulator of the CaN pathway (Wen *et al.*, 2004).

The complete molecular repertoire that controls and signals through these molecular switches in growth cone motility has yet to be elucidated. Motile responses of growth cones to calcium dependent guidance cues in an experimental paradigm of lowered Homer expression would provide significant insights into the molecular functioning of Homer in growth cone motility. Morpholino antisense oligonucleotides provide a novel approach in regulating Homer expression. They have been used extensively in non-mammalian model systems such as *Xenopus* and zebrafish and are increasingly being adapted to mammalian cell culture systems (for reviews see Angerer and Angerer, 2004; Karkare and Bhatnagar, 2006). Advantages of morpholino knockdown include ease of delivery in cell culture and the stability and efficiency of knockdown. In addition, they have not been shown to possess any of the recently described RNAi off-target effects in spine stability and morphogenesis (Alvarez *et al.*, 2006).

The aim of this study relates directly to Homer's known interactions with key calcium regulating and signalling molecules. To address this question, the endogenous expression of Homer in DRG sensory neurons will be reduced by a specific Homer1 morpholino. To establish and identify a molecular role for Homer in the calcium signalling pathways necessary for growth cone turning, morphant growth cones were assessed for their motile responses to microgradients of calcium-dependent and –independent guidance cues. The data show that a crucial level of Homer is necessary for the attraction of growth cones to the calcium-dependent guidance cues BDNF and netrin-1. Furthermore, the attractive responses of growth cones to BDNF are mediated through a CaMKII signalling pathway. In addition, the expression level of Homer in the growth cone regulated the activation of a CaN-mediated repulsive pathway.

4.2 Materials and methods

4.2.1 Cell culture

The protocol and conditions for primary culture of embryonic rat DRG neurons were identical to those described in Chapter 3.

4.2.2 Morpholino loading of DRG neurons

Loading of antisense oligonucleotide was performed using a modification of the "scrape-loading" protocol described previously (Partridge *et al.*, 1996). Briefly, oligos (5 μM) were added to dissected DRG tissue in SNM, followed by trituration until large tissue clumps were dissociated.

4.2.3 Growth cone turning assay

Turning assays were performed as described previously (Chapter 3), with the following modification(s). Pharmacological agents were added to SNM 20 min prior to commencement of imaging and remained in culture medium for the duration of the turning assay.

4.2.4 Immunofluorescence staining

Embryonic rat DRG neuron cultures were fixed in 4% paraformaldehyde at room temperature for 4 hr followed by permeabilisation and blocking with 0.4% (%/v) Triton X-100 (Sigma) and 10% (%/v) goat serum. The primary antibody, Homer1b/c (1:100-500, Santa Cruz Biotechnology) was added to coverslips overnight at 4°C. Negative control coverslips were assayed in parallel by omitting primary antisera (data not shown). Primary antibody was detected

using fluorescently labelled goat anti-mouse secondary antibody (Alexafluor-488, 1:1000, Molecular Probes). Images were acquired on a Leica DB1 microscope equipped with epifluorescence. Images (8 bit monochrome) were processed using ImageJ (NIH), Adobe Photoshop CS3 and Adobe Illustrator CS3 (Adobe Systems). Images of Homer knockdown immunostaining were acquired at identical exposure times and images were adjusted to identical greyscale threshold levels.

4.2.5 Protein sample preparation

Cells from a human neuroblastoma cell line (B-35, ATCC) were loaded with control and Homer1 morpholinos (5 μM) in an identical method to that used for DRG neurons. Following incubation at 37°C for 12 or 24 hr, cells were harvested, lysed into RIPA buffer (50 mM Tris ph 7.4, 150 mM NaCl. 1 mM PMSF, 1 mM, EDTA, aprotinin (5 μg/ml), leupeptin (5 μg/ml), 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS; all reagents from Sigma, USA).

4.2.6 Protein quantitation

Protein samples were assayed for total protein concentration using a commercial detergent-compatible total protein assay kit (D_C Protein Reagent Kit, Bio-Rad, CA, USA). Briefly, protein samples in RIPA buffer were diluted I in 5 with water then 25 μl were added to 125 μl of Reagent A followed by 1 ml of reagent B. Samples were mixed then incubated at room temperature for 15 min and absorbances measured spectrophotometrically at 750 nm. Unknown absorbances were compared to absorbances derived from a set of albumin standards (bovine albumin Type V, Sigma) assayed in the same protocol.

4.2.7 Protein electrophoresis

30 µg total protein was separated on 12% poly acrylamide gel (SDS-PAGE) then electro-blotted for 2 hr onto 0.2 µm PVDF membranes then blocked overnight in blocking solution (0.5% skin milk powder). Primary antibodies, Homer1b/c (1:1000, a generous gift of Paul Worley, Johns Hopkins, MD, USA) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, Sigma-Aldrich), were incubated for 24 hr at 4°C, rinsed thoroughly, then detected with goat anti-mouse-HRP or goat anti-rabbit-HRP secondary antibodies for 2-3hr at room temperature. Antibody conjugates were detected using ECL© chemiluminescence reagent (Pierce, IL, USA).

4.2.8 Reagents

Control (TGgTGAAcATAcGTTGTTgCCCgAT) and specific Homer1 (TGCTGAAGATAGTTGTTCCCCCAT) morpholinos labelled with either fluorescein (FITC) or Biotin were purchased from GeneTools (OR, USA). Sequences were designed to inhibit the translation of all *homer1* isoforms (ie Homer1a, 1b and 1c): KN-93 and KN-92 were purchased from Calbiochem; nerve growth factor-7S (NGF) and Cyclosporin A was purchased from Sigma-Aldrich, USA; sema-3A and netrin-1 from R&D Systems, USA; BDNF from Alomone Labs, Israel.

4.3 Results

4.3.1 Homer1b/c expression is efficiently down-regulated in rat DRG growth cones by a specific Homer1 morpholino

To assess the constitutive level of Homer expression in embryonic rat DRG neurons, wild-type DRG neurons were immunostained with an antibody directed against long-form Homer1 (Fig. 4.1). Growth cones showed a punctate distribution of Homer1b/c immunoreactivity throughout the growth cone and distal axon shaft. Puncta were also prominent along the entire axon shaft, the distal filopodial tips and axon back-branches. Similarly, in growth cones loaded with the control mismatch Homer1 morpholino, robust expression of Homer1b/c was seen throughout the entire growth cone (Fig 4.2A). Treatment of DRG neurons with a specific Homer1 morpholino however, significantly reduced Homer1b/c expression in isolated growth cones after 6 hr *in vitro* (Fig 4.2B). To assess the effectiveness of Homer1 down-regulation, a human neuroblastoma cell line (B-35, ATCC, VA,USA) was treated with the same morpholino for 6 or 24 hr. Protein extracts were probed with a polyclonal Homer1b/c antibody and showed a decrease in Homer1b/c expression by 6 hr and significant down-regulation in these cells by 24 hr, confirming that the Homer1 morpholino effectively down-regulates Homer expression (Fig 4.2C).

4.3.2 Homer1 is crucial for calcium-dependent growth cone turning

To determine if Homer1 function is required in calcium signalling, turning responses of control and Homer1 morphant growth cones to microgradients of the calcium-dependent cues BDNF, netrin-1 and the calcium-independent cue sema-3a were assessed. Significantly, DRG neurons treated with the Homer1 morpholino showed a reversal of motility from attraction to repulsion in response to microgradients of BDNF and netrin-1 (Fig 4.3). Treatment with the Homer1 morpholino, however, had no effect on DRG neuron turning in response to Sema-3a (Fig 4.3). The control morpholino had no effect on turning responses to BDNF, netrin-1 or Sema3a, and the responses were essentially identical to wild-type motile responses (compare Figs 4.3 and 3.7). Neither control nor Homer1 morpholinos significantly altered overall axon extension rates compared to untreated, or wild-type DRG neurons (compare Figs 4.3 and 3.7), demonstrating that the introduction of morpholinos into the cytosol and Homer1 knock-down does not interfere with or alter any downstream cytoskeletal rearrangements necessary for axon growth. These results strongly suggest a role for Homer1 in calcium signalling of DRG growth cone turning in response to microgradients of BDNF and netrin-1.

4.3.3 Homer1 expression alters the operational state of a CaMKII-CaN molecular switch

Since Homer1 knockdown reversed growth cone responses from attraction to repulsion to the calcium-dependent cues BDNF and netrin-1 (Fig 4.3), the question arose as to whether Homer acts through the CaMKII/CaN switch. If this were the case, inhibition of CaMKII would be predicted to have no effect on Homer morphant repulsion in response to BDNF, as attraction to BDNF is mediated by the CaMKII pathway (Wen *et al.*, 2004). Accordingly, inhibition of CaMKII with bath application of KN-93 had no effect on Homer1 morphant turning (Fig 4.4). However, control morphant attraction to BDNF was reversed from attraction to repulsion by KN-93 (Fig 4.4). The inactive analog KN-92, had no effect on control or Homer1 morphant turning in response to BDNF, in accordance with previous studies (Wen *et al.*, 2004). Inhibition of CaMKII by KN-93 did not change growth cone repulsion to sema-3A gradients irrespective of Homer expression (Fig 4.4). The pharmacological interventions outlined above did not affect overall axon extension rate, confirming that inhibition of CaMKII did not have any non-specific effects on cytoskeletal rearrangements within the growth cone. These experiments support the hypothesis that a crucial level of Homer expression is necessary for CaMKII-mediated attractive turning towards BDNF.

Homer1 knockdown resulted in reversal of growth cone responses to BDNF, suggesting that the operational state of the CaMKII/CaN switch had been modified. To test whether this reversal was due to the activation of CaN, bath application of cyclosporin A (CsA, 10 nM), an inhibitor of CaN, was used with control and Homer1 morphant DRG neurons in the growth cone turning assay. CsA application to Homer morphant cells abolished growth cone repulsion to

BDNF, resulting in random turning (Fig 4.5). This result supports the hypothesis that in the presence of reduced Homer1 expression, the CaN pathway was activated in response to BDNF signalling. Control morphant growth cone responses to BDNF were not affected by CsA treatment (Fig 4.5), as attraction is dependent on the activation of CaMKII (Wen *et al.*, 2004).

CsA treatment had no effect on sema-3a turning irrespective of Homer expression, confirming that Homer is not involved in calcium-independent turning (Fig. 4.5). As was the case in the KN-93 experiments, pharmacological inhibition of CaN did not affect overall axon extension rate, confirming that inhibition of the CaN did not have any non-specific effects on cytoskeletal rearrangements within the growth cone (Fig 4.5).

These data suggest that a crucial level of Homer1 expression is necessary for attractive turning towards BDNF, and when Homer1 is reduced, a repulsive CaN-mediated signalling pathway is activated in DRG growth cones. Taken together, these results place Homer in a key calcium-mediated signalling pathway in motile growth cones.

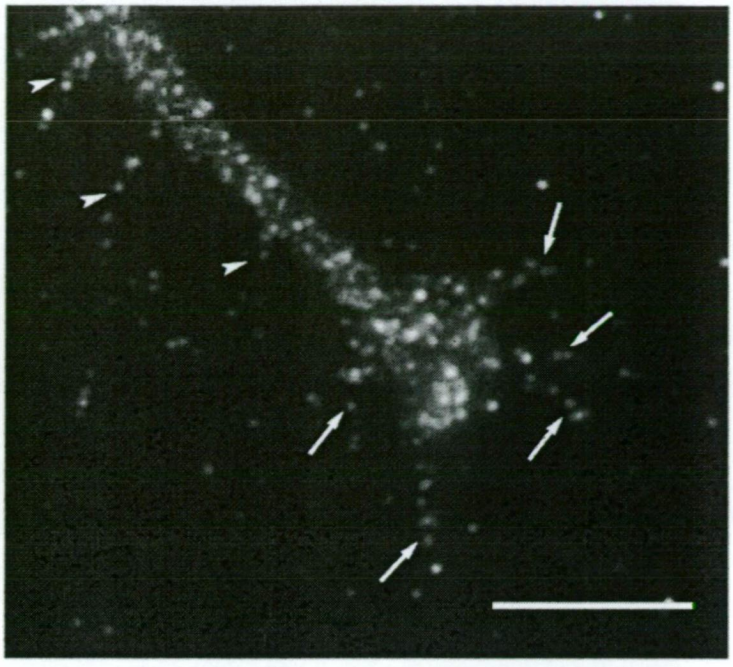


Figure 4.1

Homer is constitutively expressed in rat DRG growth cones

Figure 4.1

Homer is constitutively expressed in rat DRG growth cones

Fluorescence image of a wild-type rat DRG growth cone in culture immunostained for Homer1b/c. There was strong expression of Homer1b/c with prominent puncta over the entire surface of the growth cone and axonal shaft. Homer1b/c puncta were also prominent in the distal portions of filopodia (arrows) and several axonal back-branches (arrowheads). Scale bar is 10 μm.

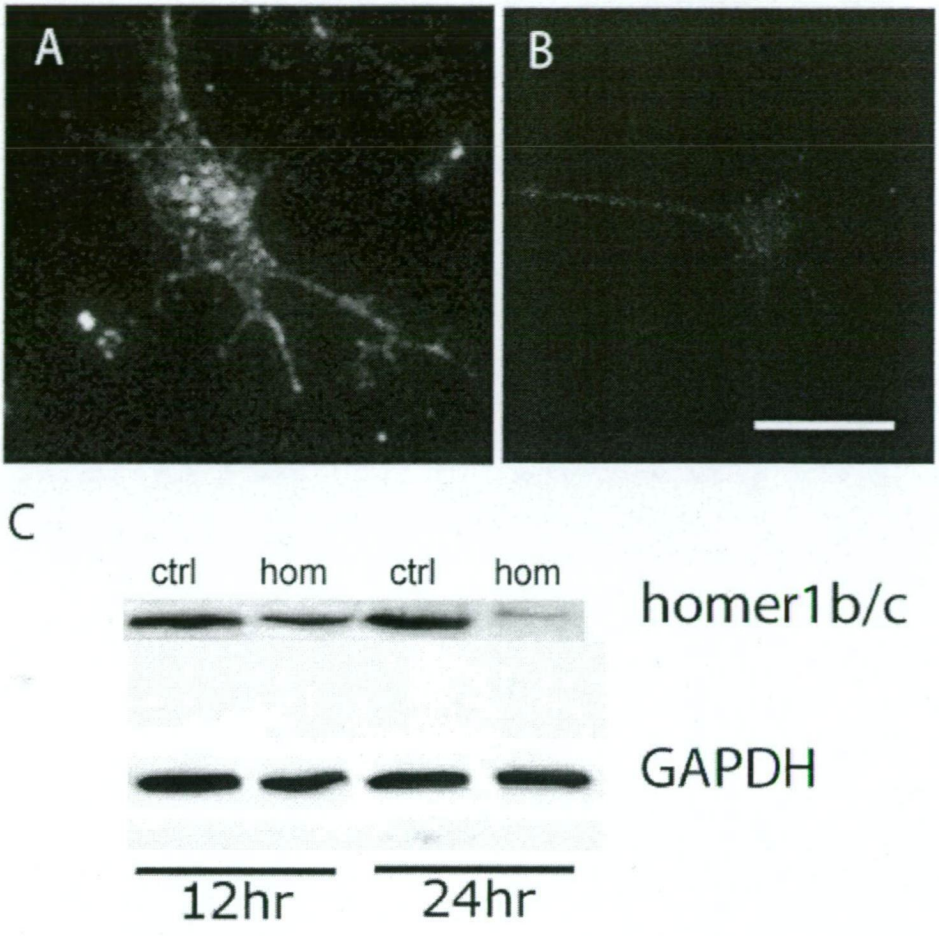


Figure 4.2

Homer knock-down by a specific antisense morpholino

Figure 4.2

Homer knock-down by a specific antisense morpholino

Immunocytochemistry and Western analysis was used to detect Homer1b/c expression after antisense morpholino treatment. Control morphant (**A**) and Homer1 morphant (**B**) growth cones labelled for Homer1b/c expression after 6 hr in culture. (**C**) The reduction in Homer expression by the specific morpholino was characterised further by quantifying Homer knock-down in a human neuroblastoma cell line (B-35). Western blot of protein extracts of B-35 neuroblastoma cells loaded with control (ctrl), and Homer1 (hom) morpholinos. Significant knockdown is achieved at 12 hr with almost complete knockdown at 24hr. Scale bar is 10 μm.

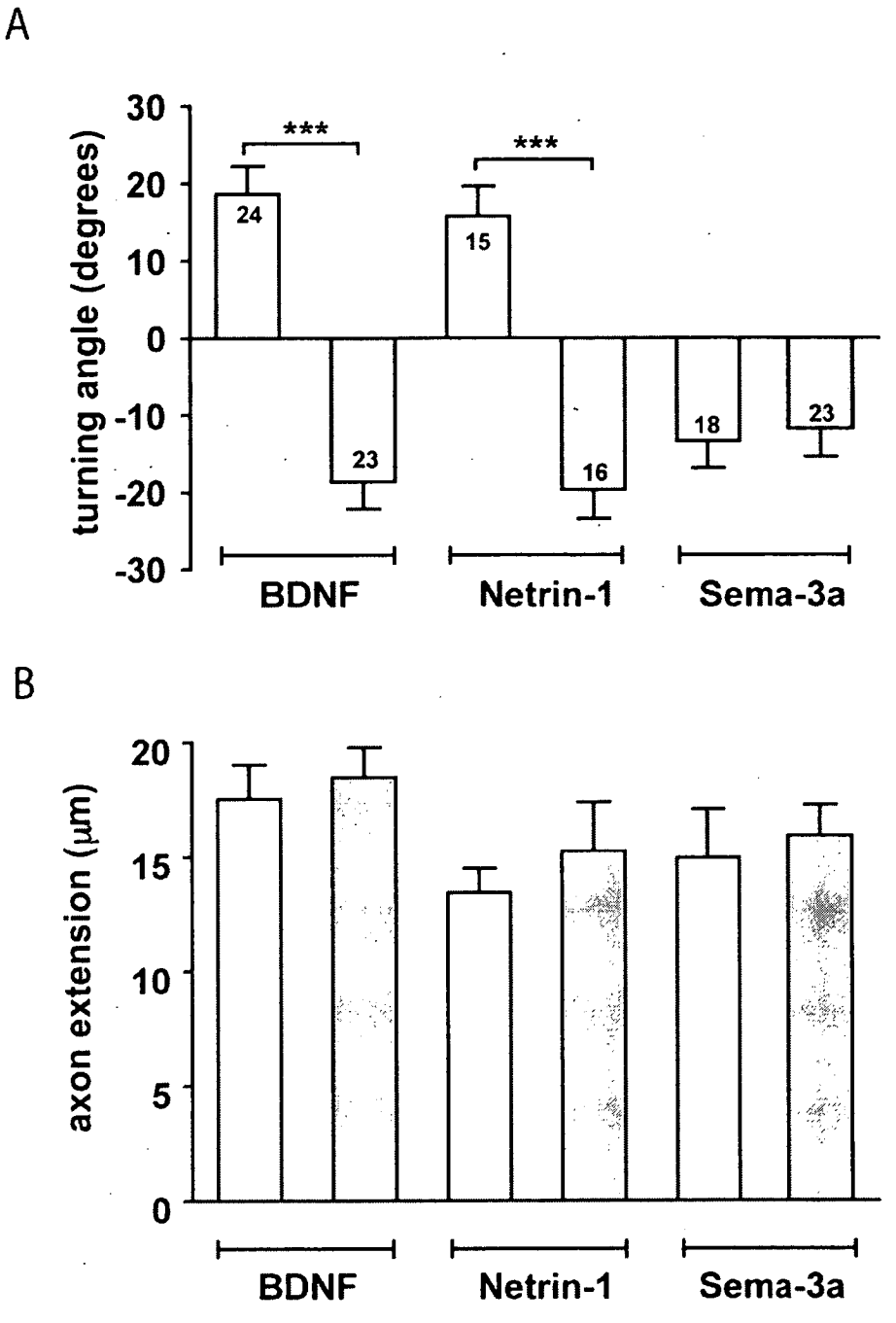


Figure 4.3

Knockdown of Homer1 reversed DRG growth cone turning from attraction to repulsion

Figure 4.3

Knockdown of Homer1 reversed DRG growth cone turning from attraction to repulsion

Motile responses of morphant DRG growth cones in the turning assay. DRG growth cones treated with a control morpholino show attractive turning towards BDNF (10μg/ml) and netrin-1 (5μg/ml) but repulsion to sema-3a (20μg/ml). In DRG neurons treated with a specific Homer1 morpholino, responses to the calcium-dependent cues BDNF and netrin-1 are reversed while repulsion to the calcium-independent cue sema-3a remains unchanged. (**A**) Average turning angles after 30 min in gradients of netrin-1, BDNF or sema-3a with 4-6 hr treatment of control morpholino (open bars) or Homer1 morpholino (shaded bars). (**B**) Control or Homer1 morpholino did not have any significant effect on overall axon extension rates (compare with Fig 3.7C). Positive angles represent attraction. Negative angles represent repulsion. Numbers in bars represent numbers of cells assayed (n). Significant differences from control values are marked as: *** $p < 0.0005$; Mann-Whitney *U*-test. Error bars indicate SEM.

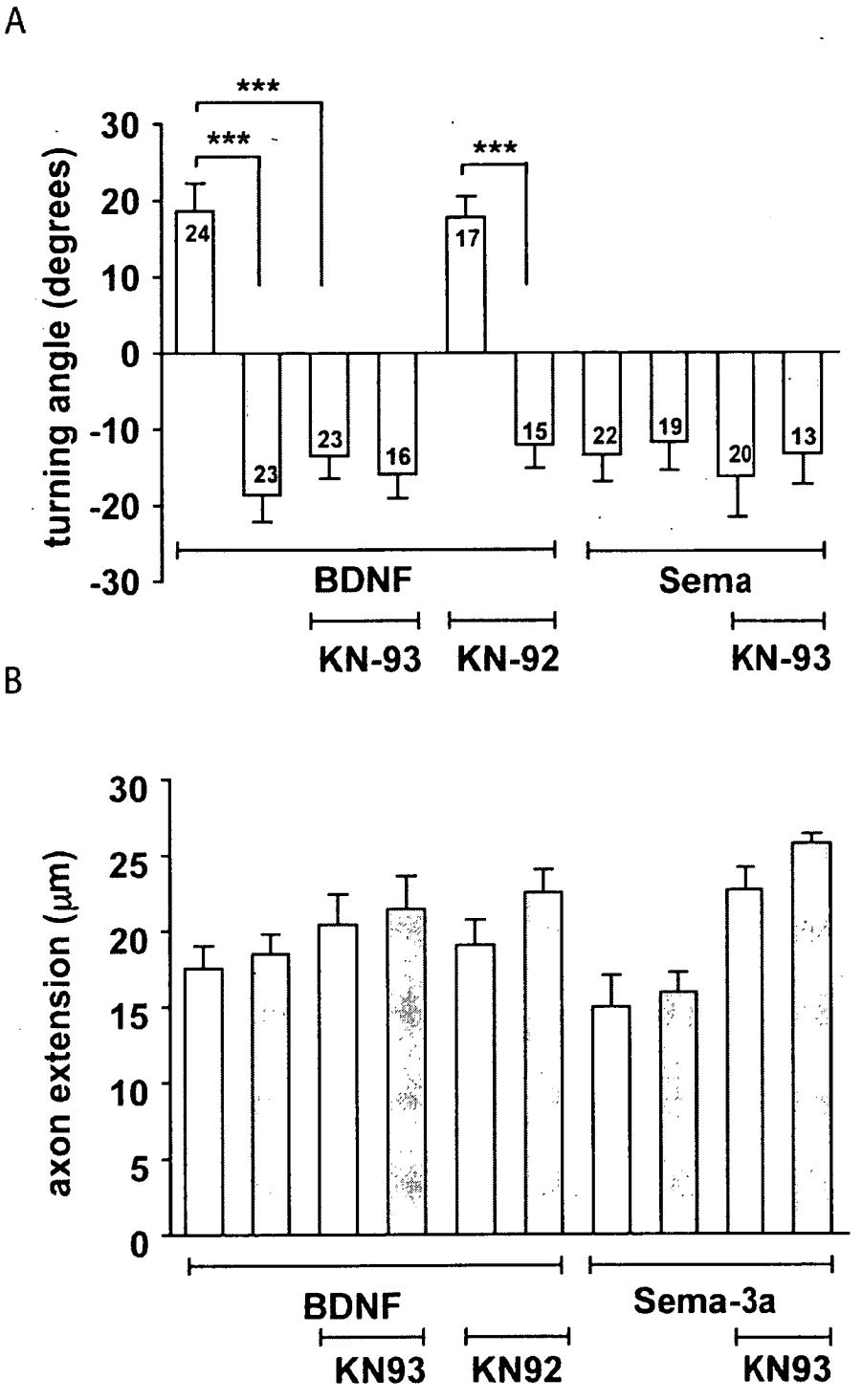


Figure 4.4

Homer knockdown blocks CaMKII activation, converting attraction to repulsion

Figure 4.4

Homer knockdown blocks CaMKII activation, converting attraction to repulsion

Motile responses of morphant DRG growth cones after bath application with the specific inhibitor of CaMKII, KN-93, or the inactive analogue, KN-92. (**A**) Average turning angles for control (open bars) and Homer (shaded bars) morphant DRG neurons in response to BDNF (10μg/ml) and sema-3a (20μg/ml) gradients following 20 min bath application of KN-93 or its inactive analogue, KN-92. (**B**) Axon extension rates were not significantly different amongst control and Homer1 morphants with the same pharmacological treatment. Significant differences from control values are marked as: ** $p < 0.005$, *** $p < 0.0005$; Mann-Whitney U-test. Error bars indicate SEM. Numbers in bars represent numbers of cells assayed (n).

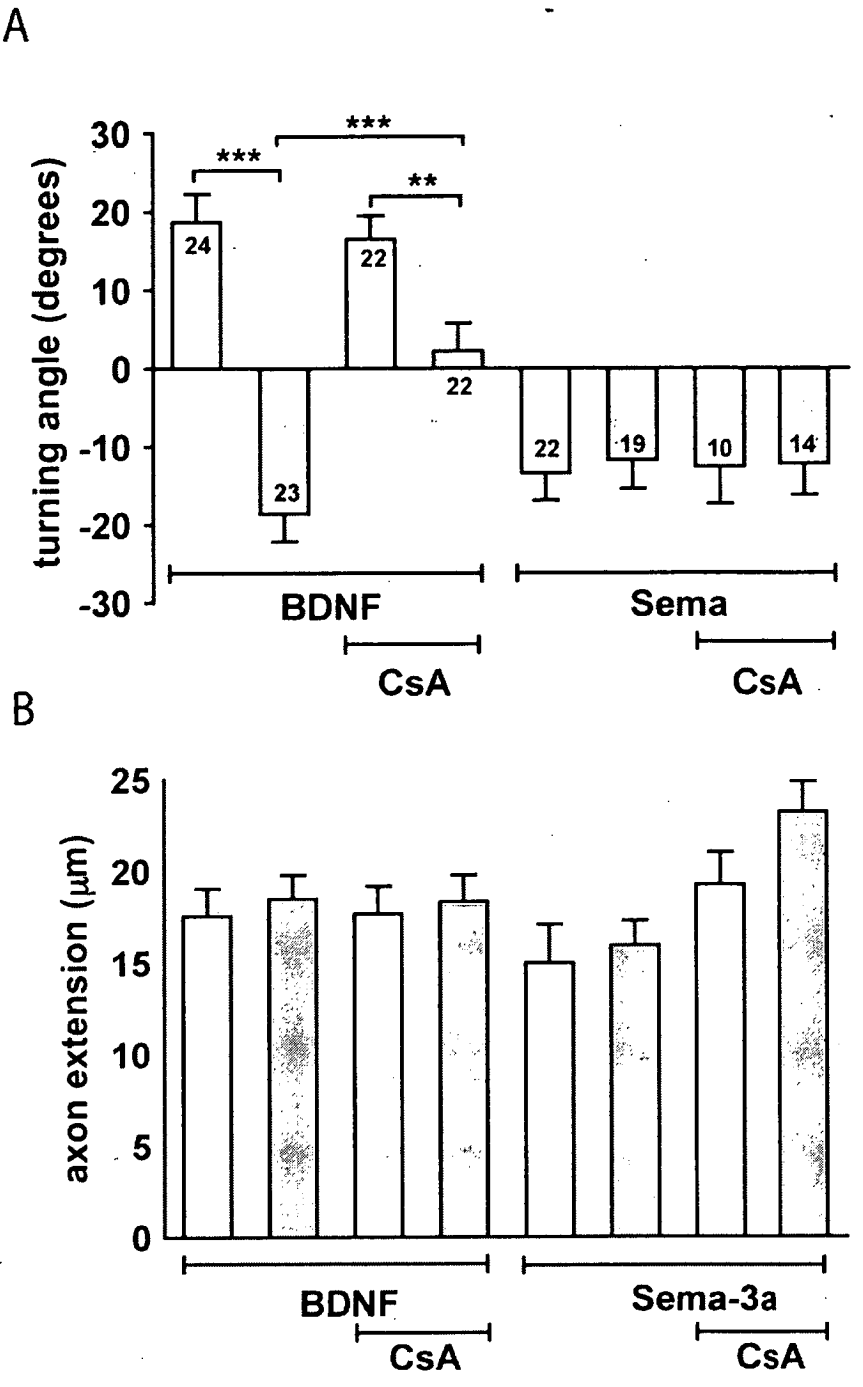


Figure 4.5

Homer knockdown activates CaN-mediated repulsion

Figure 4.5

Homer knockdown activates CaN-mediated repulsion

Motile responses of morphant DRG growth cones after bath application with CsA, an inhibitor of CaN. (**A**) Average turning angles for control (open bars) and Homer (shaded bars) morphant DRG neurons in response to BDNF (10μg/ml) and sema-3a (20μg/ml) gradients following 20min bath application of cyclosporin A. (**B**) Axon extension rates are not significantly different amongst control and Homer1 morphants with the same pharmacological treatment. Significant differences from control values are marked as: ** $p < 0.005$, *** $p < 0.0005$; Mann-Whitney U-test. Error bars indicate SEM. Numbers in bars represent numbers of cells assayed (n).

4.4 Discussion

Homer is known to be necessary for axon pathfinding *in vivo*. The experiments described in this chapter clearly demonstrate that a crucial level of Homer1 protein is required for calcium-dependent motility in embryonic DRG growth cones. Furthermore, these data provide evidence that the operational state of the CaMKII-CaN molecular switch is contingent upon constitutive Homer1 expression enabling BDNF-mediated, calcium-dependent signalling to proceed through a CaMKII-mediated attractive pathway. In the absence of constitutive levels of Homer1, motile responses to BDNF are diverted to a CaN-mediated repulsive pathway.

Constitutive expression of long form Homer1 is widespread in rat DRG growth cones, being prominent in the central area, axon shaft and filopodia. Significantly, Homer is prominent in the distal portions of filopodia and axonal back-branches. These structures are predicted to be areas of active signalling from guidance cues through to cytoskeletal structures and have been shown previously to be areas of high calcium flux and the initiation point for subsequent motile responses in the growth cone (Davenport *et al.*, 1993; Gomez *et al.*, 2001). This spatial localisation supports the developing notion that Homer is involved in the transduction of extracellular guidance signals to the growth cone cytosol, cytoskeletal elements and ultimately motile activity.

The targeted knockdown approach to gene regulation using morpholino oligos has been widely used in mammalian and non-mammalian experimental models. The approach is extensively used in *Xenopus* and zebrafish, and with evidence of specific non-target effects of RNAi to neural processes such as dendritic arbor formation and localisation of receptors (Alvarez *et al.*, 2006), is

a relevant technique to utilise in studying growth cone guidance. Using this approach, Homer1 knockdown reversed growth cone turning from attraction to repulsion in response to the calcium-dependent guidance cues netrin-1 and BDNF. It did not simply change turning from attraction to random growth. This reversal is significant and suggests that Homer modulates, or signals through, one of the proposed molecular switches that controls growth cone turning to calcium dependent guidance cues, the cAMP and/or the CaMKII-CaN molecular switches (Ming *et al.*, 1997; Song *et al.*, 1997; Wen *et al.*, 2004). These switches are not mutually exclusive and further pharmacological analysis, including competitive inhibition of cAMP and or protein kinase A (PKA), would be required to completely understand the relative contribution of each of these mechanisms, indeed the observation that cAMP directly alters the functioning of voltage-gated calcium channels (Nishiyama *et al.*, 2003) would place cyclic nucleotides upstream of calcium effectors in controlling growth cone motility.

The prominent reversal of Homer1 morphant responses suggests that a crucial level of Homer is necessary for attraction to BDNF and that Homer knockdown acted as a switch, not a dose-dependent attenuation of attraction or repulsion. To assess if Homer1 knockdown activated the recently described CaMKII/CaN molecular switch (Wen *et al.*, 2004), inhibition of CaMKII and CaN were used in conjunction with Homer knockdown in the turning assay. Inhibition of CaMKII with KN-93 had no effect on Homer1 morphant turning to BDNF and not surprisingly, it reversed control morphant responses to BDNF from attraction to repulsion since attraction is mediated by CaMKII (Wen *et al.*, 2004). Significantly, this effect was a complete reversal of attraction suggesting that once CaMKII-mediated attraction has been

abolished, a repulsive pathway was initiated, again suggestive of a switch-like mechanism.

The absence of any change to Homer1 morphant responses to BDNF when CaMKII was abolished suggests that Homer1 knockdown did not activate CaMKII but preferentially activated the CaN repulsive pathway. To test this hypothesis, CsA was used to inhibit CaN activity. Not surprisingly, control morphant responses to BDNF were unchanged when CsA was bath applied to DRG neurons in the turning assay, since attraction to BDNF is mediated by CaMKII. Homer1 morphant growth cones, however, displayed random turning when CaN was inhibited. This result is significant in that it confirms Homer knockdown activates CaN, thus mediating repulsion, but it also suggests that relieving CaN repulsion, on its own, was not able to rescue CaMKII-mediated attraction to BDNF, emphasising the requirement for Homer in CaMKII-mediated attraction. Homer1 knockdown is expected to reduce expression of all *homer1* isoforms, including Homer1a. Since Homer1a is not expressed constitutively and has only been demonstrated in activity-induced tissues, it is unlikely to play any part in such a mechanism. Indeed, if Homer1a induction is a component of turning responses, it would be predicted to act as a negative regulator of Homer1b/c.

A role for Homer1 acting directly on either CaMKII or calcineurin has not been demonstrated thus far, however Homer proteins (including Homer1a) and CaMKII are important components of the PSD (Henning *et al.*, 2007; Petralia *et al.*, 2005; Schratt *et al.*, 2004). Significantly, the responses of Homer1 morphants to sema-3a confirmed that Homer function in DRG growth cones is restricted to calcium-dependent pathways. The calcium "set point" hypothesis (Chapter 1) of growth cone motility predicts that an optimal level of $[Ca^{++}]_i$ is

necessary for appropriate responses of growth cones to guidance cues (Davenport *et al.*, 1996; Henley and Poo, 2004; Kater and Mills, 1991). The operational dynamics of the CaMKII/CaN molecular switch are sensitive to both basal $[Ca^{++}]_i$ and the depth of calcium signalling gradients (Wen *et al.*, 2004). Therefore, if the level of Homer expression regulates the operational state of such a switch, Homer1 knockdown would be predicted to regulate the dynamic nature of calcium signalling in the growth cone.

Chapter 5

Homer knockdown alters the calcium dynamics of motile growth cones

5.1 Introduction

Cytosolic calcium is a key signalling molecule regulating growth cone motility and axon pathfinding (Ghosh and Greenberg, 1995; Kater and Mills, 1991). The release of calcium from intracellular stores or influx from receptor mediated or voltage-gated channels leads to discrete $[Ca^{++}]_i$ transients and gradients, the frequency and magnitude of which correlate with overall axon growth and extension *in vivo* and directional control of responses to soluble guidance cues and axon branching *in vitro* (Gomez *et al.*, 2001; Gomez and Spitzer, 1999; Hutchins and Kalil, 2008; Zheng *et al.*, 1996; Zheng *et al.*, 1996). The calcium "set point" hypothesis (Davenport *et al.*, 1996; Henley and Poo, 2004; Kater and Mills, 1991) predicts baseline cytosolic calcium and/or frequency of transients are maintained at low levels, in order for discrete and/or global changes in $[Ca^{++}]_i$ to be detected and be instructional to growth cone motility. Clearly then, mechanisms that control and direct calcium-dependent turning will ultimately be reflected in changes in calcium dynamics in the growth cone.

TRPC channels are important non-voltage gated cation channels with a demonstrated role in regulating growth cone motility to BDNF and netrin-1 induced attraction (Li *et al.*, 2005; Wang and Poo, 2005). Furthermore, Homer is known to couple TRPC with IP_3R, regulating calcium influx and store filling in non-neuronal cells (Yuan *et al.*, 2003). Homer's demonstrated binding properties with these key calcium regulatory partners therefore, makes it a potentially important molecule in the facilitation of calcium signalling in the growth cone.

Previously in this study, targeted morpholino knockdown of Homer1 resulted in a reversal of calcium-dependent DRG growth cone turning. The question arises therefore as to whether this reversal is a consequence of changes in cytosolic calcium dynamics. It is not known how constitutive Homer functions in growth cones, however its known functions at the PSD in conjunction with its demonstrated *in vitro* functions with TRP channels and IP_3R, would predict important functions for Homer in growth cone calcium dynamics. Homer may function cooperatively with TRPC channels to regulate calcium influx and release of store calcium. Such a role would have profound implications for the control of basal $[Ca^{++}]_i$, the spatio-temporal patterning of calcium gradients and by inference, the growth cone calcium set-point. In previous experiments (Chapter 4), Homer knockdown profoundly altered DRG growth cone responses to the calcium-dependent guidance cue, BDNF, placing Homer signalling in the regulation of growth cone calcium. Inhibition of CaN abolished the reversal of growth cone responses to BDNF in morphant growth cones, suggesting that Homer function prevents CaN-mediated repulsive pathway. It is known that CaMKII-mediated growth cone attraction signals through large gradients of $[Ca^{2+}]_i$ (Wen *et al.*, 2004). It would be predicted, therefore, that calcium dynamics in Homer morphant growth cones would be significantly reduced in response to gradients of BDNF.

To determine if Homer regulates calcium signalling, single wavelength calcium imaging was used in conjunction with the already described growth cone turning assay (Chapter 3) and pharmacological manipulations of growth cones to assess whether calcium dynamics are perturbed in growth cones that have lowered expression of Homer. The effect of Homer expression on growth cone calcium dynamics was assessed using single wavelength calcium imaging. This technique, using the calcium fluorophore, Fluo-4AM, provides a

measure of changes in cytosolic calcium concentration in cells. Using this technique, wild-type DRG growth cones showed a variety of calcium dynamics, similar to those seen in other cell types (Gomez *et al.*, 2001; Gomez and Spitzer, 1999; Hutchins and Kalil, 2008). Significantly though, Homer morphant growth cones showed significant perturbations in BDNF mediated calcium release and increased frequency of spontaneous, transient calcium events, or noise. Taken together with data that showed that a crucial level of Homer expression is necessary for calcium-dependent turning (Chapter 4). There is strong evidence to suggest that Homer functions to regulate intracellular calcium dynamics, placing Homer as a key regulator of the calcium set-point.

5.2 Materials and methods

5.2.1 Cell culture

Conditions and techniques for the primary culture of embryonic rat DRG sensory neurons were as described previously in Chapter 3.

5.2.2 Immunocytochemistry

Embryonic rat DRG cultures were fixed in 4% ($^{v}/_{v}$) paraformaldehyde at room temperature for 4 hr followed by permeabilisation and blocking with 0.4% ($^{v}/_{v}$) Triton X-100 and 10% ($^{v}/_{v}$) goat serum. Primary antibodies, Homer1b/c (1:100-500, Santa Cruz Biotechnology), TRPC1, TRPC3 and TRPC6 (1:100, Alomone Labs) were added to coverslips and incubated overnight at 4°C. Detection of primary antibodies was performed using fluorescently labelled goat anti-mouse or goat anti-rabbit antibodies (Alexa-488, Alexa-594, Molecular Probes). TRPC1 and Homer1 knockdown images were acquired on a Leica DB1 microscope equipped with epifluorescence. Homer, TRPC3 and TRPC6 images were acquired with an Olympus IX80 inverted microscope equipped with a DSU spinning-disk confocal option. Fluorescence images (8-bit greyscale) were processed using ImageJ (NIH), Adobe Photoshop CS3 and Adobe Illustrator CS3 (Adobe Systems). Images of Homer, TRPC3 and TRPC6 immunostaining were acquired at similar exposure times. Subsequently, images were adjusted to identical greyscale threshold levels and converted to red-green merged images.

5.2.3 Growth cone turning assay

Turning assays were performed as previously described (Chapter 3) with the following modification(s). Pharmacological agents were added to SNM 20 min prior to commencement of imaging and remained in culture medium for the duration of the imaging or turning periods.

5.2.4 Calcium imaging

DRG neurons were plated in the previously described manner. 4-6 hr after plating, cells were loaded with Fluo-4AM calcium indicator (1μM, Molecular Probes) in artificial cerebrospinal fluid (ACSF, 137 mM NaCl, 5 mM KCl, 5.6 mM glucose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 20 mM) 0.6 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 1.4 mM $CaCl_2$, 0.9 mM $MgCl_2$) for 7 min at 37 °C, washed with fresh ACSF and incubated at 37°C for a further 15-20 min prior to imaging. Orientation of micro-pipettes and establishment of guidance cue microgradients were identical to those of the turning assay, the only difference being that an upright microscope (Leica DBM5) configuration was used in conjunction with a 20x dipping lens. Images (5-25msec exposure) were captured every 3 sec using a cooled CCD camera (ORCA, Hamamatsu) and fluorescence intensities were analysed using ImageJ (NIH) and custom software applications (Matlab, Mathworks). Calcium activity in all figures was scaled according to the following formula: $(F-F_o)/F_o$ This was calculated as the average pixel intensity (F) in a specified region-of-interest (ROI) of the growth cone divided by the baseline fluorescence intensity in the same ROI at the commencement of the observation period (F_o). All growth cone calcium measurements (except Figure 5.2) were calculated with an ROI

covering the entire growth cone area. Background fluorescence was subtracted from all frames using the average pixel value of an identically shaped ROI close to the growth cone being observed. Since the experimental configuration used in this study did not permit the quantitation of calcium activity, calcium imaging figures have been annotated with an intensity calibration bar showing the relative intensity of calcium activity (high calcium activity = white/yellow and low calcium activity = blue/magenta). Spontaneous event frequencies were calculated using a modified Daubechies 4 discrete wavelet transformation and analysis algorithm (MatLab, Mathworks).

5.3 Results

5.3.1 Calcium imaging of wild-type growth cones

Single wavelength calcium imaging using Fluo-4AM provides sufficient dynamic range to observe rapid and discrete changes in spatio-temporal concentration of calcium. Analysis of $[Ca^{++}]_i$ changes in wild-type growth cones undergoing random, non-directed protrusion and growth revealed many calcium signals or "events". The calcium signals were variable in nature, frequency and spatial distribution over the surface of the growth cone. Limitations of the imaging configuration used in this study resulted in an inability to directly observe many of the fine, motile filopodia that were present on growth cones. The events, however, were consistently observed at lamellipodial edges, growth cone transitional and central area and on several occasions, distal filopodial tips. Many events were restricted to small areas of low-level activity interspersed with flashes or pulses of activity (Fig 5.1). Calcium activity within the central area of growth cones growing in a non-directed manner, but presumably influenced by random guidance cues, was often distributed asymmetrically, with larger, more global events observed over the entire growth cone (Fig 5.2). An example of such an event is shown in Fig 5.2 with large fluctuations of calcium activity present in both ROIs at approximately 200 and 275 sec. Changes in calcium concentration were observed as discrete or global events of varying duration (Figs 5.3 & 5.4). Global events, or those involving the majority of the growth cone area appeared to be longer in duration, consisting of multiple foci of activity and relatively random in nature with growth cones undergoing periods (2-3 min) of quiescence. Filopodial tips sometimes became the focus of very rapid calcium events. These events have been described previously and are correlated with

direction of growth cone protrusion (Gomez *et al.*, 2001). Isolated, non directed wild-type DRG growth cones in the process of turning randomly showed similar behaviour. Significantly, calcium activity occurred at the distal tip of a filopodium having a trajectory approximating direction of the turn (Fig 5.5).

5.3.2 Homer knockdown alters calcium dynamics in response to BDNF signalling

The operational state of the CaMKII-CaN molecular switch is dependent on Homer expression (Chapter 4). Hence, it would be predicted that calcium dynamics within turning growth cones are perturbed in Homer1 morphants. Both Homer1 and control morphant DRG growth cones were imaged while exposed to a BDNF microgradient, similar to conditions existing in the turning assay. In control morphants, there was a robust increase in calcium flux within one minute of exposure to BDNF (Fig 5.6 A&C), consistent with the results from other groups (Li *et al.*, 2005; Wang and Poo, 2005). This produced a large global change in $[Ca^{++}]_i$ producing a steep signalling gradient. Due to hardware limitations, the length of image acquisition was not sufficiently long enough to determine whether attraction to the BDNF source was the ultimate motile response in these cells. However, previous descriptions of similar experiments using rat cerebellar granule cells confirmed that significant calcium release accompanies attractive BDNF signalling (Li *et al.*, 2005). In Homer1 morphants, the BDNF-induced rise in calcium flux was dramatically reduced (Figs 5.6 B&C). Analysis of changes in $[Ca^{++}]_i$ in multiple cells confirmed that treatment with the Homer1 morpholino almost abolished the BDNF induced rise in $[Ca^{++}]_i$ observed in DRG growth cones treated with the control morpholino (Fig 5.6 C).

Growth cone turning in response to microgradients requires release of calcium from intracellular stores (Gomez *et al.*, 2001). In order to determine if Homer1 morphants possessed functional or filled calcium stores, acute bath application of thapsigargin (50 nM) was used in conjunction with exposure to a microgradient of BDNF. In control morphants, thapsigargin did not elicit any further rise in $[Ca^{++}]_i$, confirming store release of calcium in response to BDNF microgradients (Fig 5.6 D). Homer1 morphants, however, showed a robust thapsigargin-induced increase in $[Ca^{2+}]_i$ (Fig. 5.6 E), suggesting that knockdown of Homer1 did not interfere with filling of or release from calcium stores. Hence, the lack of a $[Ca^{2+}]_i$ increase in response to BDNF gradient was not due to store depletion or non specific or off-target mechanisms affecting store function. This result suggests that constitutive Homer expression is necessary for the BDNF-induced release of calcium from intracellular stores, that is, Homer facilitates the transduction of BDNF signalling to calcium stores.

5.3.3 Homer knockdown increases the frequency of spontaneous calcium events in DRG growth cones

Homer knockdown profoundly altered calcium dynamics in DRG growth cones in response to a BDNF micro-gradient. A closer analysis of calcium responses prior to the establishment of a BDNF gradient revealed a significant increase in the frequency of spontaneous calcium transients in Homer1 morphants (Figs 5.7 B&D) compared to control morphants (Figs 5.7 A&C). The frequency of these transient events in control morphants was comparable to that described previously (Gomez *et al.*, 1995; Hutchins and Kalil, 2008). These results demonstrate that Homer1 protein has a crucial function in the attenuation of spontaneous calcium transient frequencies in motile DRG growth cones.

5.3.4 Calcium transients in Homer1 morphants are derived from store operated calcium channels.

Depending on the cell type, it is generally accepted that transient calcium activity is not due to the activity of voltage gated calcium channels (Gomez *et al.*, 2001; Gomez *et al.*, 1995; Gomez and Spitzer, 1999; Lautermilch and Spitzer, 2000; Tang *et al.*, 2003; Ziv and Spira, 1997). To determine if transient calcium events are due to the activity of store calcium channels, the relatively non-specific inhibitor of store-operated channels, SKF96365 (Merritt *et al.*, 1990), was bath applied to Homer1 morphant cells in the calcium imaging protocol. The increase in frequency of spontaneous events in Homer morphant cells observed was significantly attenuated with SKF96365 to levels

seen in control morphant cells suggesting that transient calcium events are derived from store operated calcium channels, possibly TRPC (Figs 5.7 E&F).

Homer may control calcium transients by its demonstrated ability to bind and gate TRPC channel activity (Yuan *et al.*, 2003). Bath application of SKF96365 abolished attractive turning of DRG growth cones towards a microgradient of BDNF (Fig 5.8), suggesting that DRG growth cone turning relies on calcium influx through store-operated calcium channels. These results strongly suggest that Homer's regulation of growth cone responses to calcium dependent guidance cues is mediated by its previously described interactions with TRPC channels.

5.3.5 DRG neurons express Homer 1b/c, TRPC1, TRPC3 and TRPC6 proteins in functionally relevant areas of growth cones

In order for Homer to gate TRPC channels, thereby regulating $[Ca^{++}]_i$ in the motile growth cone, it would be predicted that Homer and TRPC proteins are localised at the leading edges of the growth cone and in other functionally relevant structures, such as filopodia. Homer1b/c is constitutively expressed in embryonic rat DRG growth cones (refer Chapter 3). Immunofluorescent staining using a specific Homer1b/c antibody showed that DRG neurons express Homer1b/c in functionally relevant areas of the growth cone such as the central area and distal tips of filopodia (Figs 5.9 A,D&G). Significantly, the complete repertoire of TRPC mRNA's have recently been demonstrated in mouse dorsal root ganglia (Elg *et al.*, 2007). Embryonic rat DRG growth cones express TRPC1, TRPC3 and TRPC6 protein ubiquitously in DRG growth cones (Figs 5.9 B,E&H). The expression pattern was similar to that of Homer with close apposition and/or colocalisation of TRPC and Homer

proteins in functionally relevant areas of the growth cone such as the central area (Figs 5.9 C,F,I&J) and filopodia (Fig 5.9 F&K). Closer analysis revealed the intimate apposition of Homer and TRPC3 protein at distal filopodial tips and along filopodial shafts (Figs 5.9 K1,K2,K3 & K4).

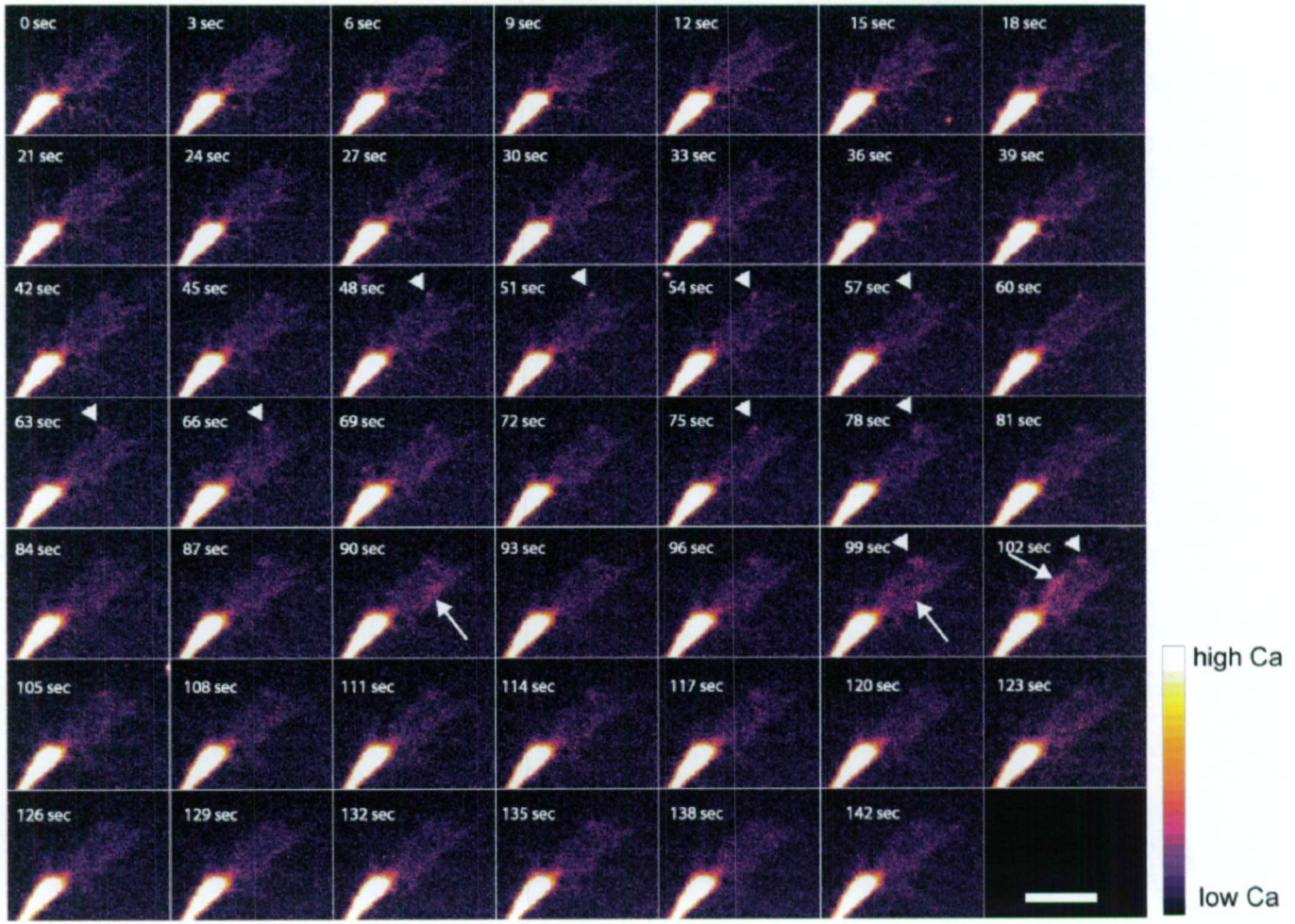


Figure 5.1 Calcium dynamics in wild-type growth cones

Figure 5.1

Calcium dynamics in wild-type growth cones

Series of time-lapse images showing calcium activity ($\Delta F/F$) of a wild-type growth cone undergoing non-directed growth and protrusion. The cell was loaded with the calcium indicator Fluo-4AM and calcium activity has been pseudo-coloured (see calibration bar, inset). Activity at the lamellipodial edges (or proximal filopodial tips) can be transient in nature. Arrowheads denote a small area of transient, but regular calcium activity. Other, more global activity is usually centred at the growth cone central area. Arrows show changes in the spatial distribution of cytosolic calcium in one region of the central area. Time interval between frames is 3 secs and scale bar is 5 μm.

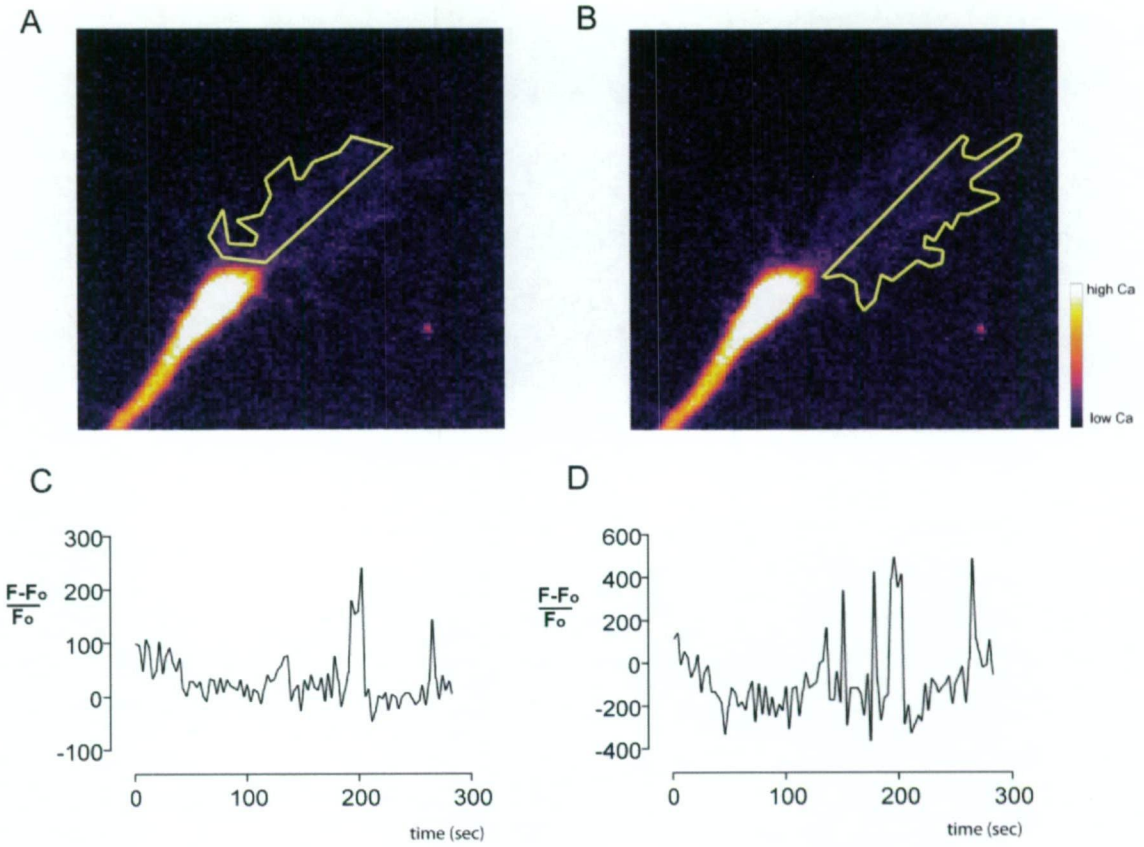


Figure 5.2 **Calcium release and gradient dynamics are asymmetric**

Figure 5.2

Calcium release and gradient dynamics are asymmetric

Time-lapse images showing calcium activity (ΔF/F) of growth cones in conditions of random, non-directed growth often show asymmetrical distribution of calcium release. Figure shows a representative frame of a calcium imaging time lapse sequence. The growth cone image stack was divided into two regions of interest (ROI) based along its longitudinal axis. Fluorescence intensities have been subsequently determined for every frame in the acquisition sequence (300 sec). (**A & B**) The growth cone and all visible filopodial structures was bisected and 2 ROI defined. (**C**) Pixel intensities for the ROI shown in (A) over 300 sec show only small amount of activity with one major event at 200 sec. (**D**) Pixel intensities for the ROI shown in (B) show greater numbers and amplitude of calcium events.

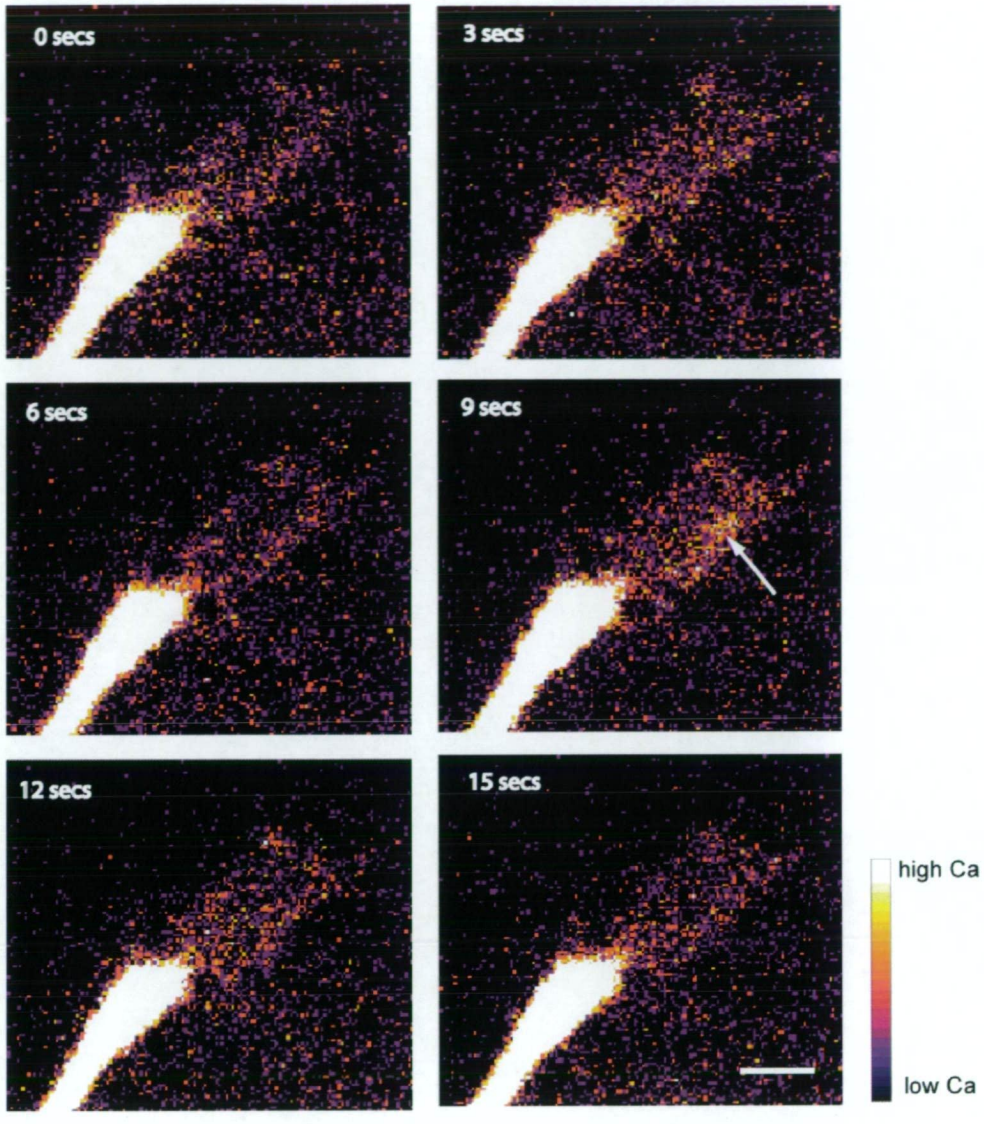


Figure 5.3

Calcium release in the growth cone central area can be discrete and short-lived

Figure 5.3

Calcium release in the growth cone central area can be discrete and short-lived

Spatial disturbances in cytosolic calcium due to calcium release and/or influx may be extremely rapid. Figure shows a sequence of fluorescence ($\Delta F/F$) images of a growth cone undergoing a discrete calcium transient event in the growth cone central area. Arrow denotes area of calcium activity that appears between 2 successive frames then dissipates rapidly. Frame interval is 3 secs. Scale bar is 5μm.

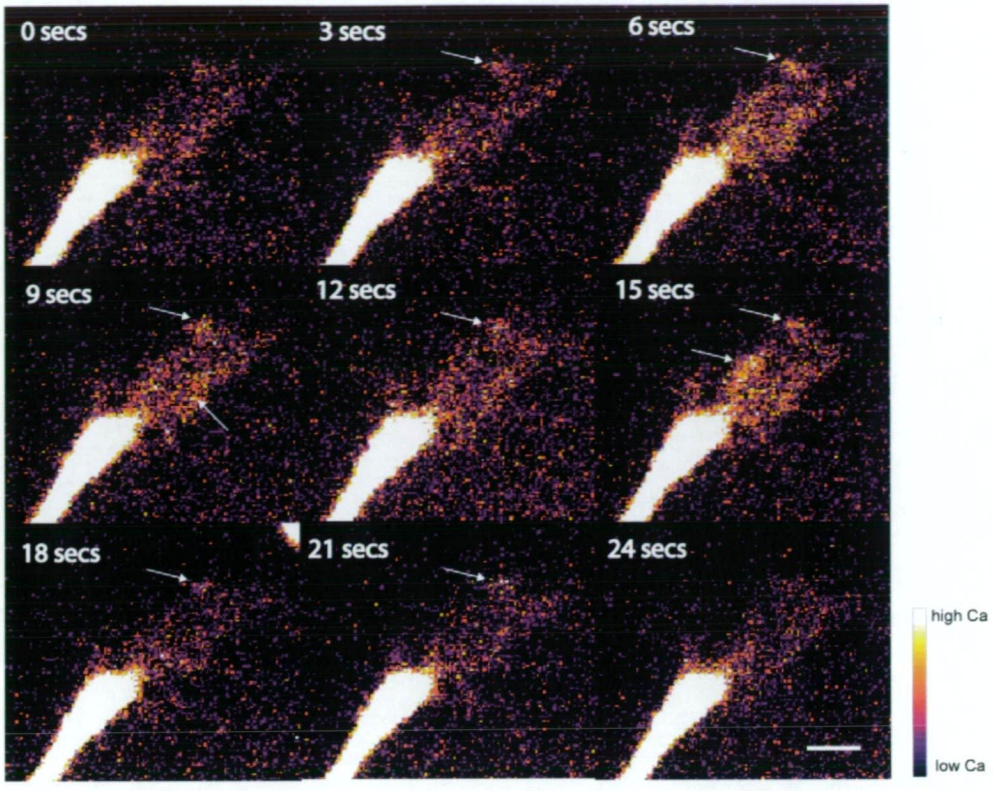


Figure 5.4

Calcium release in the central area of the growth cone can be global and long-lived

Figure 5.4

Calcium release in the central area of the growth cone can be global and long-lived.

Figure showing a sequence of fluorescence images of the growth cone shown in Fig 5.3 imaged some time later, showing another transient event, spatially restricted to a different area but with a significantly longer duration (approximately 18 sec) than in Fig 5.3. Closer examination of this global event reveals distinctly separate regions of calcium activity within the central area (arrows). Scale bar is 5μm.

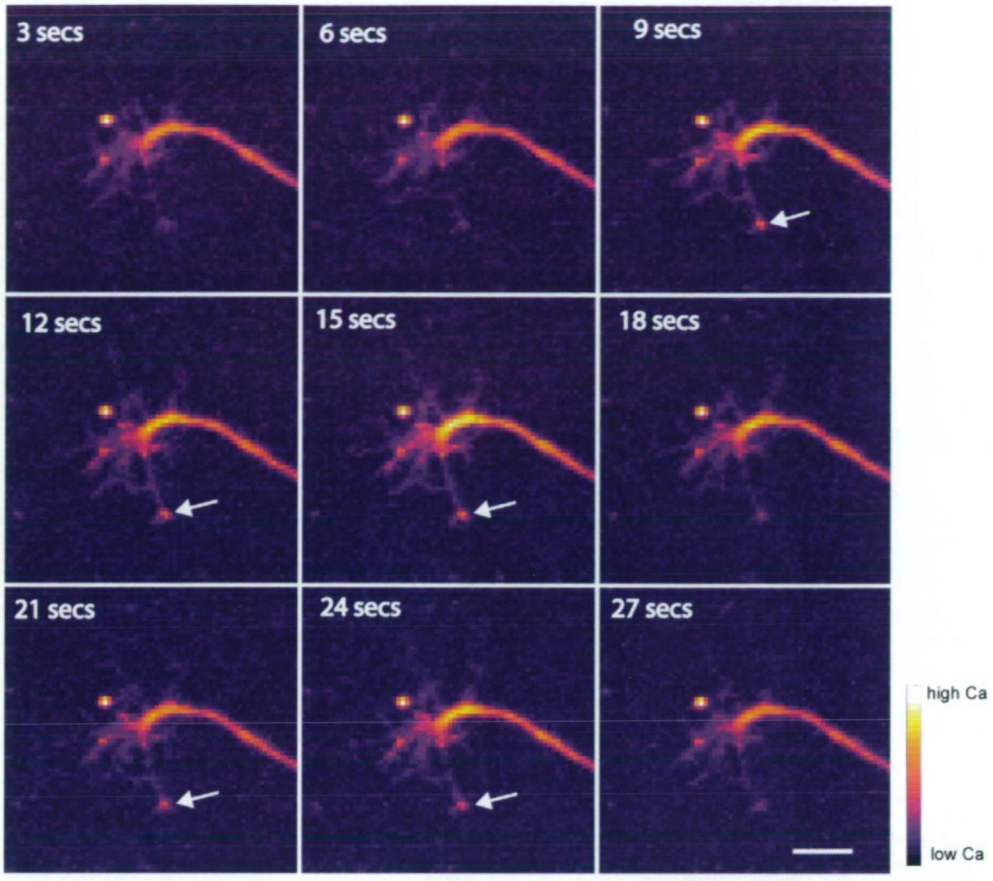


Figure 5.5 Calcium dynamics in filopodia

Figure 5.5

Calcium dynamics in filopodia

Time lapse image sequence showing a growth cone with many thin, protruding filopodia. Calcium activity in filopodia is rapid and is often restricted to the distal filopodial tips. Distal filopodial tip of filopodium marked with arrow shows two bursts of calcium release, at frames 9 through 15 sec and 21 though 24 sec. Scale bar is 5μm.

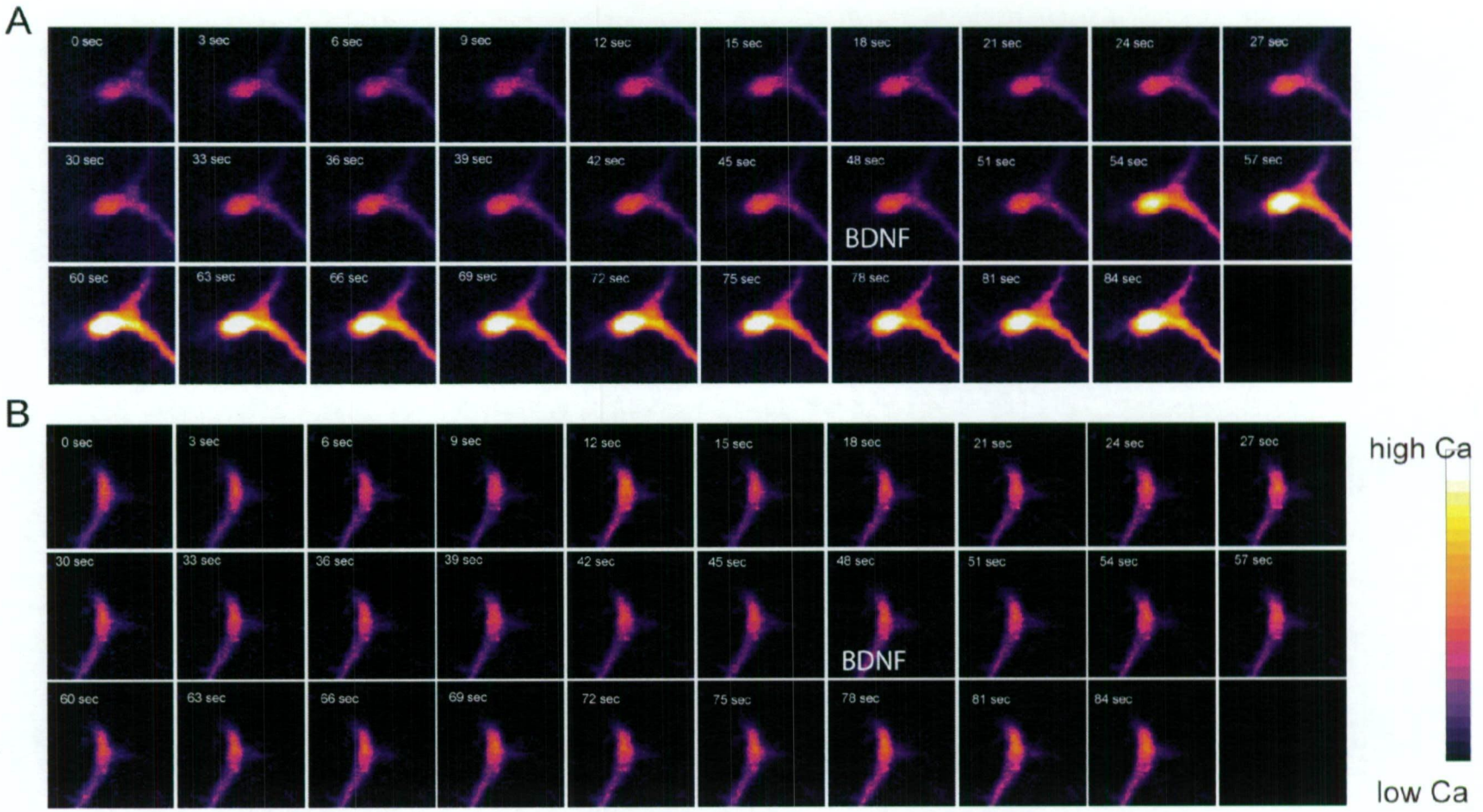


Figure 5.6 (A&B) **Homer1 knockdown perturbs growth cone calcium release**

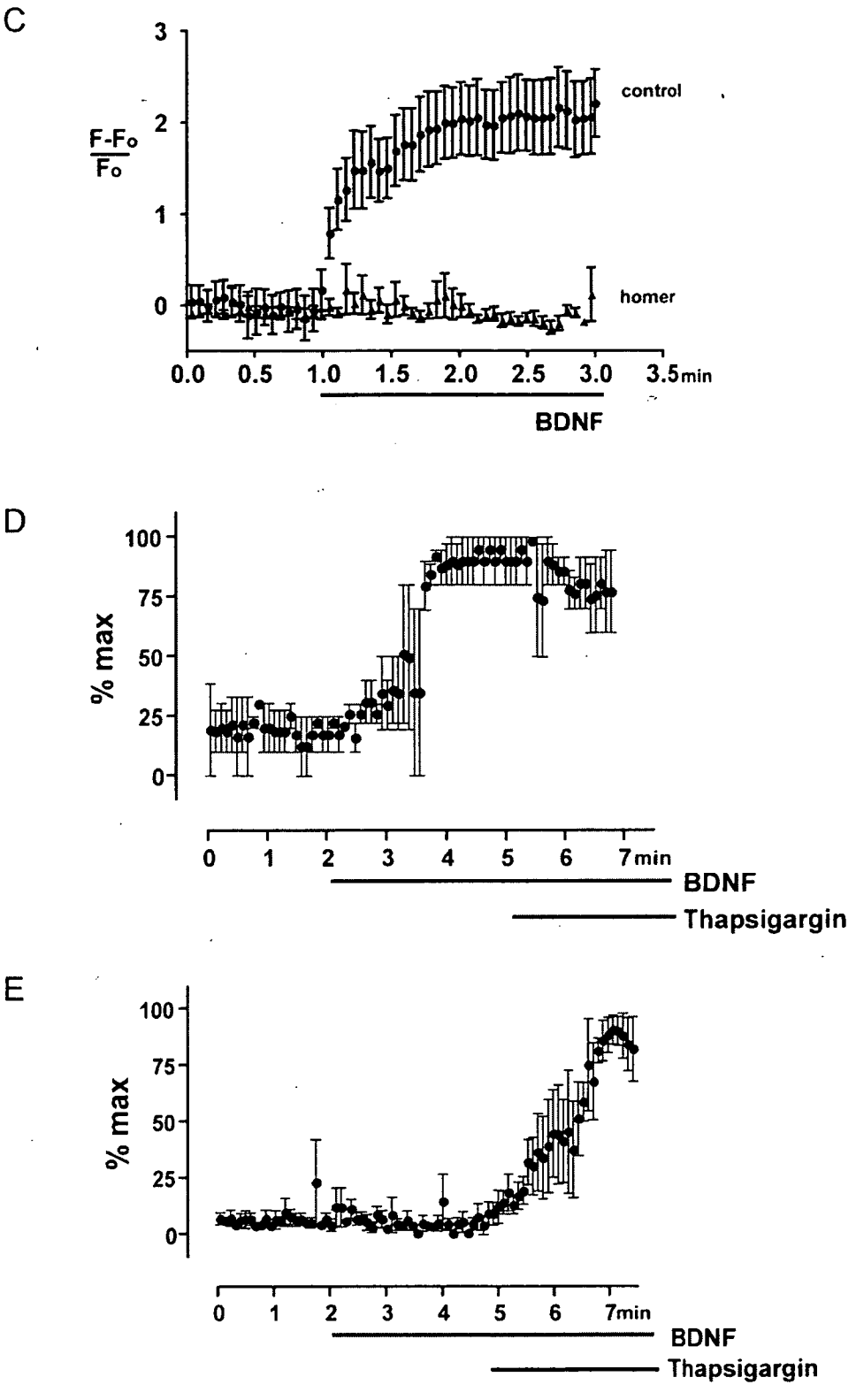


Figure 5.6 (C,D&E)

Homer1 knockdown perturbs growth cone calcium release

Figure 5.6

Homer1 knockdown perturbs growth cone calcium release

Single wavelength calcium (ΔF/F) imaging of DRG growth cones in response to a microgradient of BDNF. **(A)** Sequential images of control morphant growth cone showing robust calcium release in response to a BDNF gradient. **(B)** Homer1 morphant growth cone showing dramatic reduction of BDNF-induced calcium release. **(C)** Quantitation of the above responses. $[Ca^{++}]_i$ responses of control morphant growth cones (n=9) exposed to a BDNF microgradient show significantly more calcium release than Homer1 morphants (n=16). **(D & E)** Homer1 morphant calcium stores retain functionality. **(D)** Thapsigargin was bath-applied 2 min after establishment of BDNF microgradient. Stores were depleted after exposure to a BDNF microgradient in control morphants, with no further increase in $[Ca^{++}]_i$ after thapsigargin application (n=6). **(E)** Store calcium was not released during exposure to a BDNF microgradient in Homer1 morphants, but could be released in response to thapsigargin (n=6). Error bars in **(C)** indicate SEM. BDNF microgradient was established at "BDNF" frame in (A & B). Orientation of pipette (out of field of view) in A&B is at the upper left quadrant of the image frame. Frame interval is 18 sec.

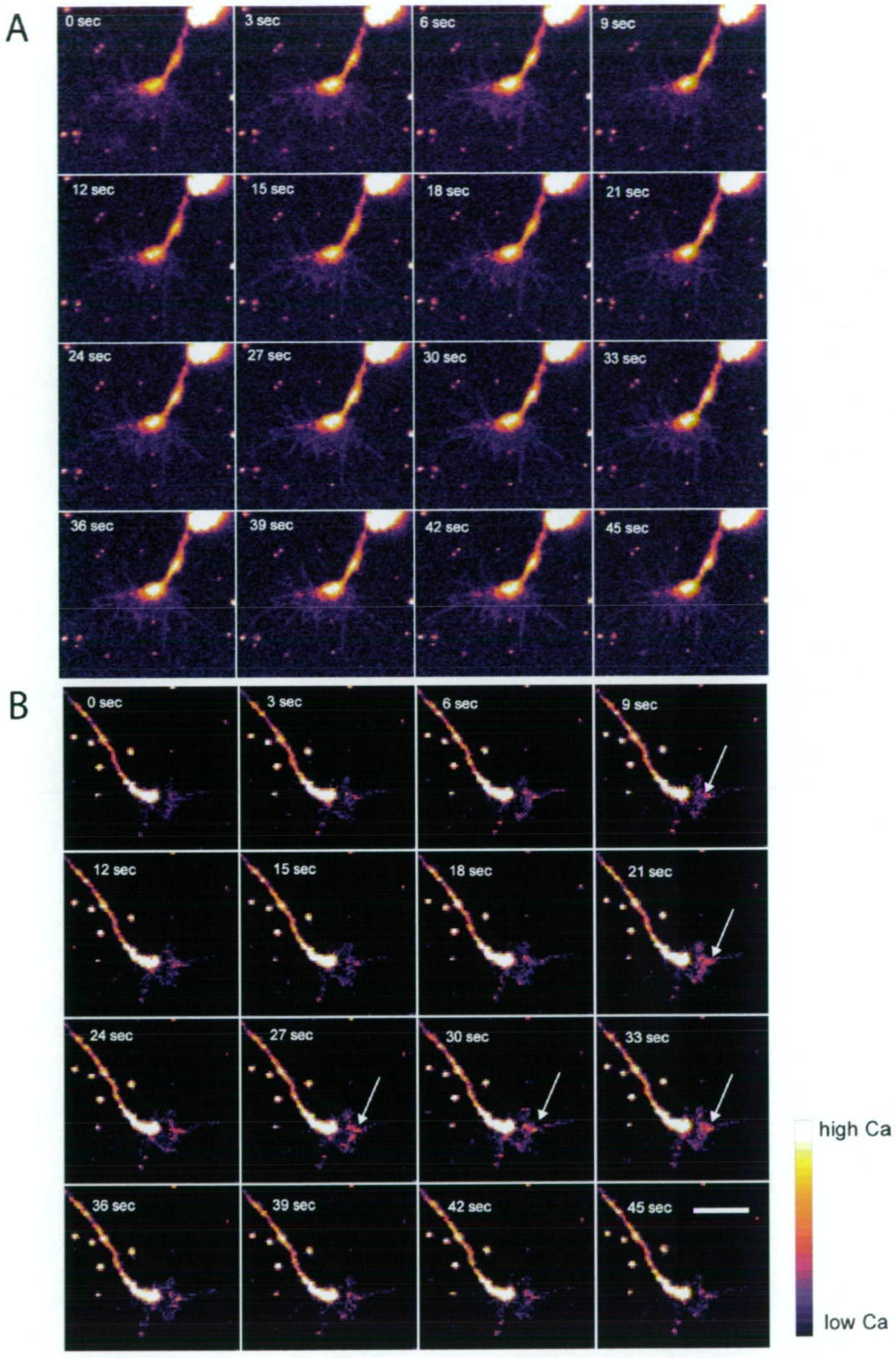


Figure 5.7 (A&B) **Homer1 knockdown increases the frequency of spontaneous calcium events**

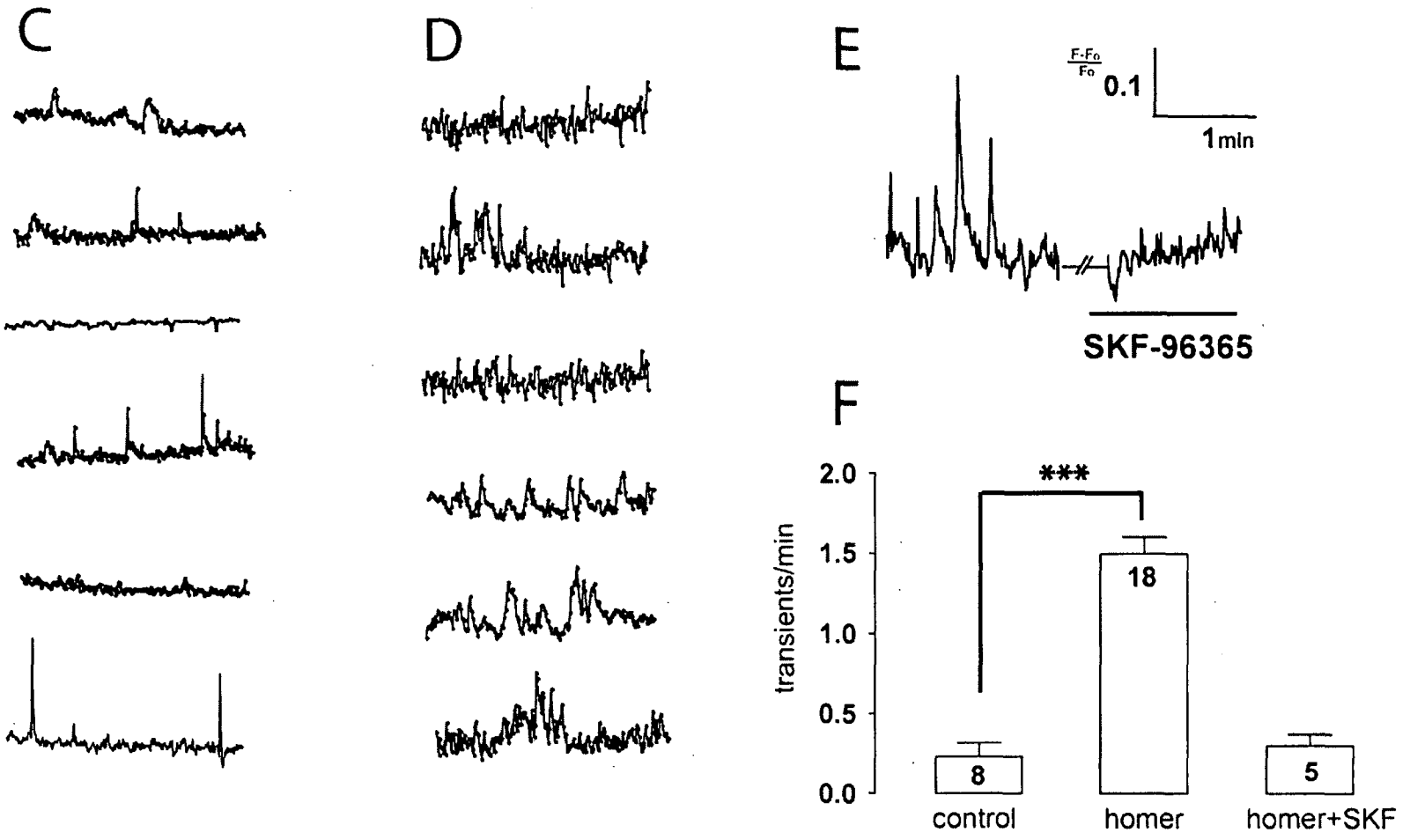


Figure 5.7 (C,D,E&F) **Homer1 knockdown increases the frequency of spontaneous calcium events**

Figure 5.7

Homer1 knockdown increases the frequency of spontaneous calcium events

(A-D) Homer1 knockdown results in an increase in SKF96365-sensitive transient calcium activity. Baseline calcium responses from an individual **(A)** and 6 separate control morphant growth cones **(C)** showing sparse transient calcium activity. Calcium responses from an individual **(B)** and 6 separate Homer1 morphant growth cones **(D)** exhibiting significantly greater frequency of spontaneous events. **(E)** Calcium responses from a single Homer1 morphant growth cone showing a decrease in transient frequency with bath application of SKF96365. **(F)** Quantitation of transient event frequencies. SKF significantly attenuates the frequency of spontaneous events in Homer1 morphant DRG growth cones. Significant differences are marked as: *** $p < 0.0005$; Mann-Whitney U-test. Error bars indicate SEM. Scale bar refers to C, D & E.

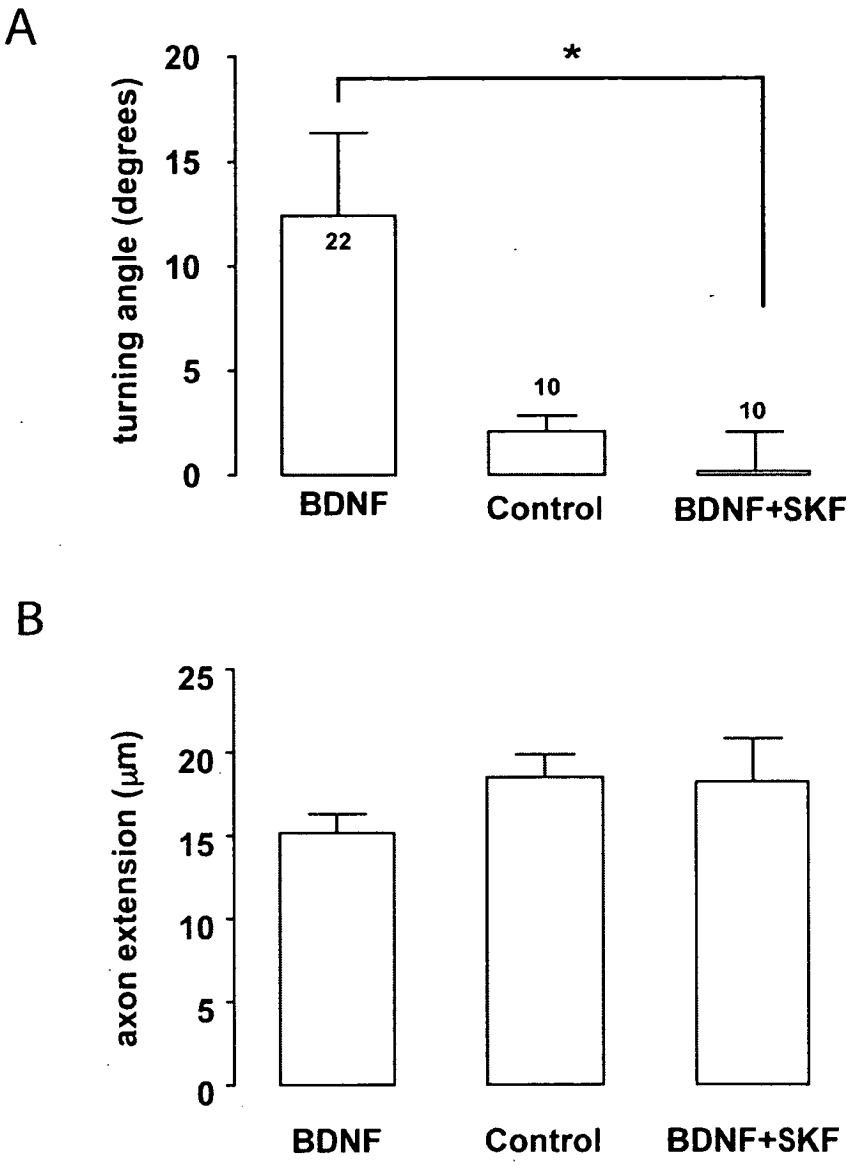


Figure 5.8

SKF96365-sensitive calcium channels are necessary for BDNF-mediated growth cone motility

Figure 5.8

SKF96365-sensitive calcium channels are necessary for BDNF-mediated growth cone motility

(A) Growth cone turning towards BDNF is abolished when SKF96365-sensitive channels are inactivated. **(B)** SKF treatment is specific to turning since axon extension rates do not differ significantly from BDNF only or control experiments. Significant differences are marked as: * $p < 0.05$; Mann-Whitney U-test. Error bars indicate SEM.

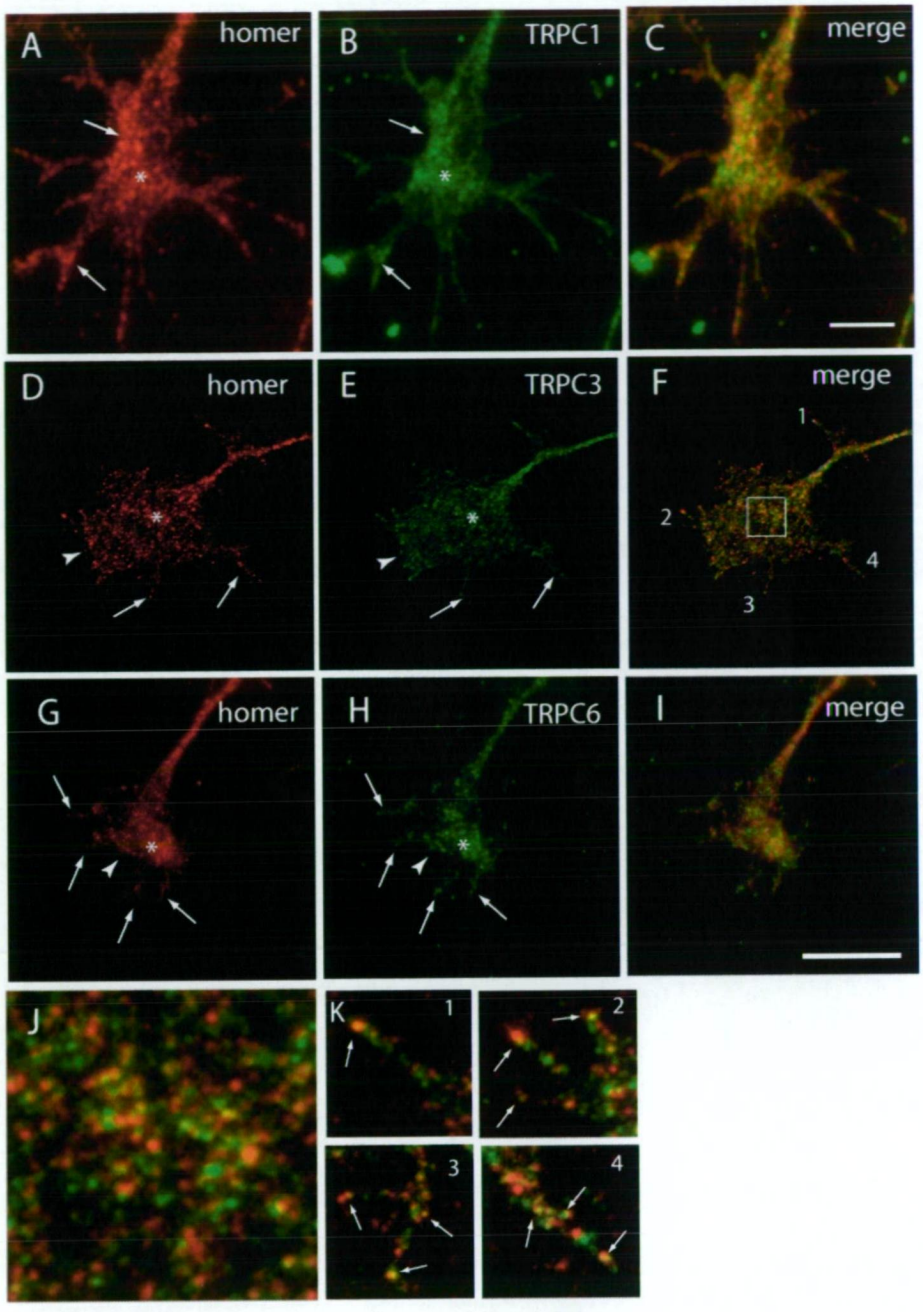


Figure 5.9

DRG growth cones express Homer 1b/c, TRPC1, TRPC3 and TRPC6 proteins in functionally relevant areas of growth cones in a pattern of close apposition and/or co-localisation.

Figure 5.9

DRG growth cones express Homer 1b/c, TRPC1, TRPC3 and TRPC6 proteins in functionally relevant areas of growth cones in a pattern of close apposition and/or co-localisation.

(**A,D&G**) Immunostaining of DRG neurons for Homer1b/c revealed robust punctate expression of Homer protein in growth cone central area (*), filopodia (arrows), and leading edges of lamellipodia (arrowhead). (**B,E&H**) Immunostaining of DRG neurons for TRPC1, TRPC3 and TRPC6 respectively, revealed a similar punctate expression pattern in growth cone central area (*) and filopodia (arrows) and leading edges of lamellipodia (arrowhead). Merged images underscore the close apposition and/or co-localisation of Homer1b/c and TRPC protein in growth cone central area (box, **F** and enlarged in **J**) and filopodia (**F**, filopodia numbered 1, 2, 3 & 4, enlarged in **K**). Scale bars: (**A-C**) 5μm; (**D-I**) 5μm

5.4 Discussion

In order to understand the mechanisms of Homer function in the growth cone, single wavelength calcium imaging was used to correlate calcium dynamics and motile growth cone behaviour. Wild-type growth cones revealed complex patterns of spatio-temporal calcium activity under conditions of random growth. Experiments described previously (Chapter 4) suggested that the reversal of turning responses to calcium-dependent guidance cues in Homer morphants was a consequence of altered calcium dynamics in the growth cone. In this study, control morphant growth cones showed robust release of store calcium in BDNF-directed turning. In Homer morphants, however, calcium responses were greatly reduced. Significantly, calcium imaging of control and morphant growth cones revealed significant increases in the frequency of SKF96365-sensitive, calcium transients in Homer morphants. These results suggest a crucial role for Homer in the calcium signalling mechanisms operating within motile DRG growth cones.

Growth cones showed minimal variation in area during imaging protocols and any significant condensations suggesting probable growth cone collapse justified the immediate exclusion of that growth cone from any further analysis. This addresses one of the main technical concerns surrounding single wavelength imaging, where changes in the volume of the growth cone and hence compartmentalisation of calcium fluorophore could invalidate the quantitation of calcium concentration by fluorescence intensity. Historically, the measurement of calcium concentration in living cells has utilised dual wavelength ratiometric measurements to compensate for variables such as uneven dye loading, cell thickness, photo-bleaching and dye leakage (Tsien and Harootunian, 1990). Single wavelength measurements, however, with

appropriate selection of fluorophore and calibration procedures are capable of generating quantitative data without the complexity of dual wavelength optics (Maravall *et al.*, 2000). Changes in growth cone volume are unlikely to bias any such measurements. Indeed, rapid changes in growth cone morphology such as collapse would be the most likely events to affect any analysis of calcium flux, and as a consequence would be rejected from analysis.

Wild-type growth cones showed a variety of calcium events during random, non-directed axon protrusion *in vitro*. The calcium events varied spatio-temporally, with short periods of quiescence interspersed with periods of activity. A feature of the more prominent periods of activity was the asymmetry of calcium distribution suggesting that even random growth is controlled by directional calcium signalling in the growth cone, consistent with that observed in directed motility (Hong *et al.*, 2000; Zheng, 2000). Growth cones extended filopodia into the culture surroundings and many of the more rapid events involved bursts of calcium activity in regions well out into the distal tips of filopodia. Generally, there was considerable variation in the spatio-temporal distribution of calcium events in wild-type growth cones, ranging from focal and rapid "sparks" to global, long-lived increases in calcium activity. Seminal work by Gomez *et al* (2001) showed that similar characteristics of filopodial calcium transients were crucial for substrate-dependent growth cone turning. Release of calcium from intracellular stores or influx from receptor-mediated or voltage-gated channels is known to result in discrete $[Ca^{++}]_i$ transients and gradients, having variable frequencies, magnitudes and a variety of spatial patterns (Gomez *et al.*, 2001; Gomez and Spitzer, 1999; Zheng *et al.*, 1996).

The observation that Homer knockdown reversed calcium dependent growth cone turning *in vitro* suggests that it is involved in the regulation of calcium dynamics within the growth cone. Indeed, the calcium imaging experiments described here revealed that calcium dynamics in Homer1 morphants were profoundly altered on two levels: a BDNF-induced rise in cytosolic calcium seen in control morphants was almost completely abolished in Homer1 morphants. In addition, and there was a significant increase in the frequency of spontaneous calcium transients in Homer1 morphants.

Calcium is released from intracellular stores in response to calcium-dependent guidance cues such as BDNF (for reviews see Gomez and Zheng, 2006; Henley and Poo, 2004). Thapsigargin is used as a store depletion agent to examine calcium release from intracellular stores. It inhibits SERCA pumps, preventing uptake of calcium into stores (Thastrup *et al.*, 1990). Acute application of thapsigargin manifests itself as a rapid increase in $[Ca^{++}]_i$ (Gomez *et al.*, 1995; Thastrup *et al.*, 1990). In control morphants, a robust, sustained rise in $[Ca^{++}]_i$ was observed in response to a gradient of BDNF. There was little or no additional calcium release with thapsigargin in control morphants, confirming efficient store calcium release in response to BDNF signalling. Homer morphants, however, showed little or no increase in $[Ca^{++}]_i$ in response to BDNF, but a robust increase in $[Ca^{++}]_i$ could be elicited by thapsigargin. This result confirmed that Homer1 knockdown did not interfere with filling of internal stores. Furthermore, this result suggests Homer1 is required to trigger store release upon BDNF signalling through TrkB receptors and subsequent TRPC activation. This hypothesis requires further investigation; however, it is consistent with the role of Homer acting to couple TRPC and IP_3R, maintaining the signalling complex in an "activation-ready" state (Yuan *et al.*, 2003).

The operational dynamics of the CaMKII/CaN molecular switch are sensitive to both baseline $[Ca^{++}]_i$ and the depth of calcium signalling gradients (Wen *et al.*, 2004). Previous work in non-neuronal cells demonstrated that Homer-mediated coupling of TRPC to store calcium via IP_3R leads to the formation of a responsive signalling complex at the cell membrane. Interruption of this complex results in TRPC channels that are spontaneously active (Yuan *et al.*, 2003). Significantly, Homer1 morphant DRG growth cones turning in response to BDNF display significant perturbations of normal calcium release dynamics, suggesting an uncoupling of the TRPC-Homer-IP_3R complex. It would be predicted therefore, that in addition to aberrant store release of calcium in response to BDNF, Homer1 morphants would show calcium events that are correlated to spontaneously active TRPC channels. Analysis of baseline calcium levels in Homer1 morphants showed a significant increase in the frequency of calcium transients compared to control morphants. Bath application of SKF-96365 significantly reduced calcium transients to control morphant levels, suggesting that the transients originate from TRPC and/or other SOC-derived signals (such as ORAI-1 (Huang *et al.*, 2006)). Previous studies have eliminated voltage-dependent calcium channels as sources of these transient events (Gomez *et al.*, 2001; Tang *et al.*, 2003). The exact identity of these SKF-sensitive transients requires further experimental elucidation, for example knockdown or silencing of TRPC in conjuction with further pharmacological experiments to exclude VGCCs will be required. These data, when taken together with that of others, suggest that TRPC cation channels are the source of transient calcium events (Li *et al.*, 2005b; Shim *et al.*, 2005; Wang and Poo, 2005; Yuan *et al.*, 2003).

DRG growth cone turning and calcium fluxes in response to BDNF were inhibited by SKF-96365 confirming a requirement for TRPC in calcium-dependent growth cone turning. These results are consistent with known function of TRPC in motile growth cones. The TRPC family of cation channels have crucial functions in growth cone turning. Calcium dependent turning of *Xenopus* spinal neurons requires xTRPC1 signalling (Shim *et al.*, 2005; Wang and Poo, 2005). In cerebellar granule cells, the calcium-dependent guidance cues BDNF and netrin-1 signal through TRPC3 and TRPC6 channels (Li *et al.*, 2005). These studies concluded that BDNF and netrin-1, via respective TrkB and DCC receptors, either directly or indirectly activate TRPC, which in turn activate calcium signaling in the growth cone (Li *et al.*, 2005; Shim *et al.*, 2005; Wang and Poo, 2005). Mutation of TRPC channels or treatment with SKF-96365 abolished calcium flux in growth cones and attractive motile responses to calcium-dependent guidance cues, while inhibition of L-type voltage-gated calcium channels had little effect on BDNF-induced turning (Li *et al.*, 2005).

Homer1b/c and TRPC cation channels interact directly *in vitro*. In HEK-293 cells, Homer1b/c couples TRPC with IP_3R (Yuan *et al.*, 2003) and in neuronal cells Homer1b/c binds the IP_3R (Tu *et al.*, 1998). In DRG growth cones therefore, it is likely that Homer1b/c binds TRPC and IP_3R, facilitating store operated calcium influx and release in response to calcium-dependent guidance cues. Dorsal root ganglia express the full complement of TRPC mRNA's in the embryonic mouse (Elg *et al.*, 2007). DRG neurons express TRPC1, TRPC3 and TRPC6 proteins in functionally relevant areas of embryonic rat growth cones. Protein expression experiments suggested that while colocalisation of TRPC with Homer1b/c was not absolute throughout the growth cone, significant colocalisation or close apposition on filopodia (especially the distal tips and proximal shafts) was observed. Initiation of

filopodial calcium transients regulates spatial and temporal qualities of global growth cone calcium signals (Gomez and Letourneau, 1994; Gomez *et al.*, 2001). Localisation and/or association of these signalling elements on crucial structures therefore, suggests that Homer-TRPC-IP_3R coupling is well-placed to transduce signals from extracellular cues to intracellular motile machinery.

Recent work by Kim *et al* (2006) has demonstrated a novel suite of interactions between Homer, TRPC3, IP_3R complexes and the plasma membrane in non-neuronal cells *in vitro*. In their quiescent or resting state the Homer1b/c-TRPC3-IP_3R complexes are located partly in the plasma membrane-ER interface and partly in intracellular vesicles. Upon depletion of ER stores by IP_3R, Homer1b/c-TRPC3-IP_3R complexes are dissociated and translocated to the cell membrane. Similarly, when Homer1b/c-TRPC3-IP_3R complexes are dissociated by introducing short form, Homer1a, TRPC3 translocation occurs rapidly at the plasma membrane where TRP channels become spontaneously active. In both of these scenarios, the Homer-TRPC3-IP_3R complexes are reconstituted upon filling of store calcium. It is this novel translocation role for Homer1b/c and TRPC that bears much significance with respect to the generation of transient calcium channel activity in the Homer1 morphant growth cones. In such a mechanism, knockdown of Homer1 would be sufficient to dissociate Homer-TRPC-IP_3R complexes and to translocate TRPC to the plasma membrane. Since the immunolocalisation was performed on resting or non directed DRG neurons, the level of colocalisation may be a reflection of the random TRPC-Homer translocation state of individual neurons. Clearly, future work in this area would involve the quantitative immunolocalisation of Homer and TRPC protein at various times points during turning in addition to using techniques aimed at uncovering rapid molecular interactions at the sub-cellular level, such as FRET.

Homer has crucial functions at the PSD. Homer clusters mGluR complexes at the plasma membrane effectively attenuating voltage-gated calcium and potassium channels in SCG neurons (Kammermeier *et al.*, 2000). Evidence presented previously (Chapter 4) strongly suggests that Homer functions with TRPC to effectively attenuate spontaneous activity of TRPC. Homer may also function cooperatively with TRPC to transduce BDNF signalling through to calcium store release. Taken together, this data supports the hypothesis that Homer effectively regulates baseline calcium activity. The integral of these perturbations in calcium activity would be predicted to elevate baseline or background $[Ca^{++}]_i$ effectively destabilising the calcium "set-point". The exact nature of the perturbation in $[Ca^{++}]_i$ was not able to be determined in the current studies due to the limitations and constraints of the imaging protocol, however, it could be predicted that a significant increase in transient activity would alter the $[Ca^{++}]_i$ signalling gradient.

From the work presented here it is likely that knockdown of Homer1 resulted in a shallow $[Ca^{++}]_i$ signalling gradient, due to disruption of the TRPC-IP_3R-Homer1 complex, resulting in disruption of signalling to ER stores, spontaneously active TRPC channels and an increase in calcium transient frequency. These results would predict that the $[Ca^{++}]_i$ gradient would be shallow, since CaN signals through such gradients (Wen *et al.*, 2004). At the present time, visualisation of $[Ca^{++}]_i$ gradients is not possible, and only inferred from focal application or release of calcium from caged compounds. Also, the contribution of voltage-gated calcium and potassium channels to the increase in transient activity requires further elucidation, including a more specific pharmacological or genomic approach to TRPC activity. Taken together with work described in Chapter 4, showing that Homer functions through the

activation state of a CaMKII/CaN molecular switch, and previous work by others (Kim *et al.*, 2006; Yuan *et al.*, 2003) showing the integral nature of cooperation between Homer and TRPC, the calcium imaging experiments described here implicate Homer as a key regulator of the intracellular "set-point", necessary to maintain accurate directional control of growth cone motility.

Chapter 6

Conclusions and Future Directions

The precision and accuracy of circuit formation is a crucial element in the development of complex neural behaviour. However, the complete repertoire of cellular events controlling this process, are still unclear. Fundamental to any such understanding are the molecular events that direct and control the motility of axonal growth cones. The work described in this thesis presents strong evidence for the role of Homer, a well characterised post-synaptic molecule, as having a key role in the pre-synaptic environment of developing axons.

Homer has previously been shown to be necessary for axon pathfinding *in vivo* in the *Xenopus* embryo. Using the developing zebrafish embryo, experiments outlined in Chapter 2 of this thesis describe the ontogeny of Homer1b/c expression in the developing sensory nervous system. Homer expression paralleled the behavioural development of the zebrafish larva, and its requirement for functional neural circuitry, to be able to interact with its environment and survive. The expression pattern seen in the zebrafish olfactory structures such as the basement membrane of the olfactory placode, though yet to be fully demonstrated, may reflect a parallel, context-dependent role for Homer in the developing zebrafish. Such expression may relate to a short-range or contact-mediated guidance role for Homer in the zebrafish early in development. The temporal correlation of Homer and sensory system development, however, is in accordance with its previously described *in vivo* pathfinding function in the *Xenopus* retinotectal system.

To more fully understand the possible mechanisms by which Homer may function in axon pathfinding, an *in vitro* approach using rat sensory neurons was used to ask whether Homer functions in the growth cone to regulate

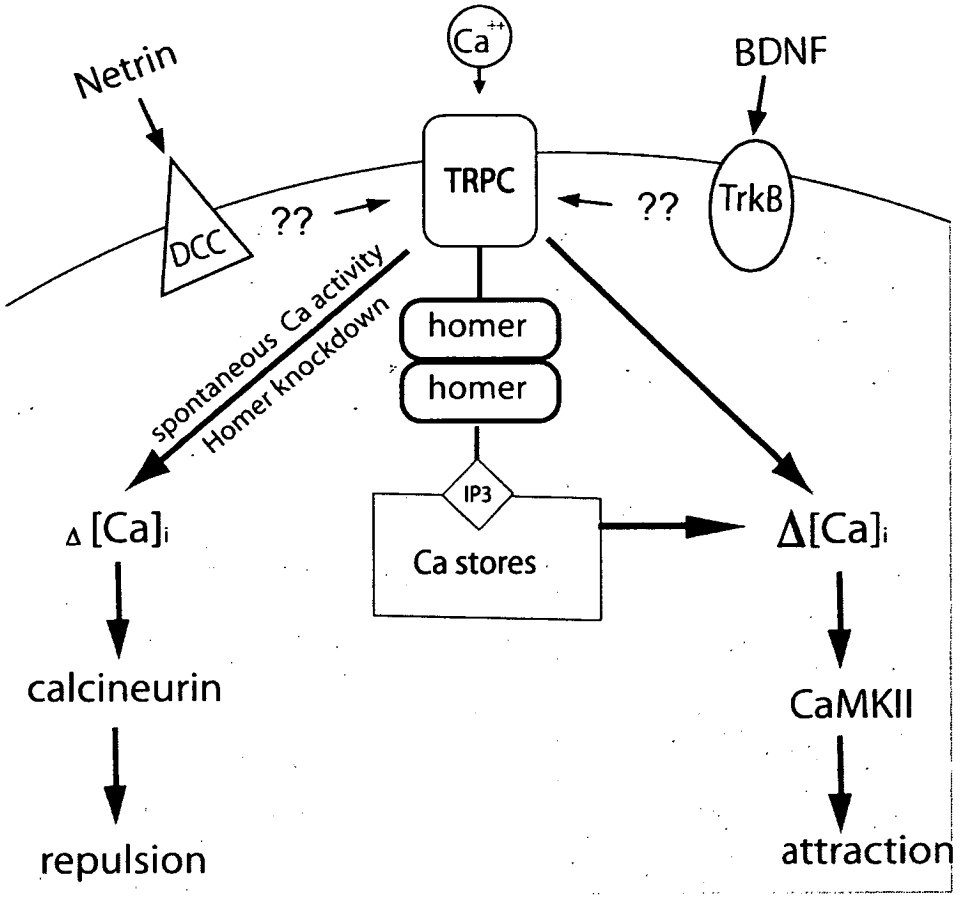
motility. To address this question an *in vitro* growth cone turning assay was developed and used to characterise the motile responses of wild-type growth cones to the important guidance cues BDNF, netrin-1 and sema-3a. The power of such an assay allows the experimenter to vary the expression of a molecule, or perturb the pharmacological environment and assess the growth cone's motile responses in a quantitative manner. This assay was used to confirm the novel chemoattractive properties of metallothionein proteins, and will be used in the future to elucidate their mechanism of action on regenerating neurons. More significantly however, turning experiments also uncovered a role for Homer in the growth cone attraction to the important calcium-dependent guidance cues BDNF and netrin-1. When constitutive Homer expression was reduced by morpholino knockdown, attractive motile responses to BDNF and netrin-1 were completely reversed. The data also demonstrated that Homer was not required for responses to the chemo-repulsive, and calcium-independent molecule, sema-3a.

Combining the turning assay with pharmacological interventions of the previously described CaMKII/CaN molecular switch, experiments described in Chapter 4 showed that a crucial level of Homer1 was required for CaMKII-mediated attractive turning towards BDNF and that Homer knockdown resulted in CaN-mediated repulsion. Significantly, these result implicate Homer function in crucial intracellular signal transduction mechanisms utilising a CaMKII/CaN molecular switch and the crucial second messenger, calcium.

Many of the molecular and cellular events controlling growth cone motility have been previously shown to use calcium as a key signal transduction molecule (Davenport *et al.*, 1996; Gomez *et al.*, 1995; Guthrie *et al.*, 1991; Lankford and Letourneau, 1989; Mattson *et al.*, 1988; Smith *et al.*, 1983).

Changes in growth cone motility following extracellular receptor/ligand signalling are ultimately transduced to rearrangements of the cytoskeletal machinery in the growth cone through changes in the spatio-temporal patterning of $[Ca^{++}]_i$. At the PSD, Homer facilitates calcium signalling through its metabotropic receptor clustering capacity and its ability to bind the IP_3R, while *in vitro*, in non-neuronal cells, Homer couples TRPC channels to IP_3R thus regulating calcium release from stores (Tu *et al.*, 1998; Xiao *et al.*, 1998; Yuan *et al.*, 2003). If Homer functions in the growth cone to regulate calcium-dependent motile events then it would be predicted to do so by controlling calcium dynamics. Experiments outlined in Chapter 5 show that constitutive long-form Homer expression acts to attenuate the spontaneous activity of TRPC channels, thereby reducing the frequency of spontaneous calcium transients, previously shown to be crucial in growth cone motility and axon pathfinding.

The following is a summary of the hypothesised molecular events in which Homer may function in the growth cone. For simplicity, it does not illustrate any of the other important calcium regulating moieties known to facilitate ER release of calcium, such as RyR, Stim1, Stim2 and Orai1.



Motile responses to the guidance cues BDNF and netrin-1 are mediated by their respective receptors, TrkB and DCC, and store operated TRPC calcium channels. The data presented here supports the hypothesis that BDNF signals through constitutive levels of a Homer1-TRPC-IP_3R complex facilitating the store release of calcium, generating a deep cytosolic calcium gradient. This is interpreted by a CaMKII/CaN molecular switch and transduced as CaMKII dependent attraction. In the event of Homer-TRPC-IP_3R complex dissociation, eg by Homer1 knock-down, store release of calcium does not proceed upon BDNF signalling (TrkB activation). Furthermore, TRPC channels become spontaneously active leading to a high frequency of transient calcium events. The experiments described in Chapter 5 however, do not completely resolve the possibility that the spontaneous activation of TRPC and calcium transient by themselves, are sufficient to disrupt IP_3-

mediated release of store calcium. The integral of transient cytosolic calcium increases due to these events subsequently raises basal $[Ca^{++}]_i$, thus producing a shallow calcium gradient which results in CaN dependent repulsion. Crucial levels of Homer therefore, facilitate the transduction of extracellular guidance cues to effect cytosolic calcium dynamic changes in the motile growth cone, ultimately controlling the calcium "set-point" of the cytosol.

Much remains to be understood regarding the dynamic nature of these molecular interactions. For calcium gradients to be instructional to the growth cone, dynamic rearrangements of receptors, signalling complexes and cytoskeletal elements need to be distributed in spatio-temporal arrangements that direct growth cone protrusion in a specified direction. In addition to its gating of TRPC channels, Homer orchestrates the translocation and redistribution of TRPC channels *in vitro*. Such dynamic interactions between crucial calcium signalling partners would be expected to have profound implications for signalling in the growth cone. It is not known if these dynamic interactions are refined by activity, developmental expression of receptor sub-types or signalling from target tissues. Given the heterogeneity of receptor subtypes on DRG neurons, the large number of demonstrated TRP channels on sensory neurons so far and their demonstrated capacity to heterodimerise, it would seem likely that the regulation of Homer-TRP interactions is far more complex that has been presented in this work (Elg *et al.*, 2007; Goswami and Hucho, 2007; Hjerling-Leffler *et al.*, 2007). Studying the development and refinement of these receptors would undoubtedly provide many insights into the general question of how sensory axons navigate such vast distances during development and function in the adult nervous system.

Other interacting partners that would be predicted to modify Homer and calcium signalling include the short form, or Homer1a. The spatio-temporal dynamics of such an interaction, if present in the growth cone, would be predicted to have significant effects on calcium gradients. Homer1a, the only activity-inducible form of Homer demonstrated so far, would effectively uncouple any Homer-TRPC-IP_3R complexes leading to spontaneously active TRPC channels, this effect however, would be in the context of neural activity. To date, the induction of Homer1a following activity in growth cones has not been demonstrated. The question could be asked whether long-form Homer-TRPC complexes develop into a different suite of receptor sub-types to adapt to a new environment of activity-dependent signalling as they approach their targets in circuit formation. Many of these questions will be addressed with new and emerging technologies such as FRET and FLIP to examine the dynamics of Homer's interactions with signalling partners in the growth cone.

The data described in this thesis supports the hypothesis that Homer can be considered a facilitator of extracellular signal transduction at the growth cone and ultimately axon targeting. Experiments described in this thesis using zebrafish larvae demonstrate that Homer protein is expressed in sensory systems at behaviourally significant time points. Coupled with its role as an important regulator of the calcium setpoint *in vitro* and its known synaptogenic functions in the circuit development of the rodent hippocampus, Homer can be seen as a facilitator in the development of complex neural circuitry. Indeed, a highly conserved molecule that is located pre- and post-synaptically, has major signal transduction functions in both of these locations and is developmentally regulated in the embryo would fulfil such a role. The challenge remains, however, to understand Homer's complete systematic and functional integration into the biochemical repertoire of the developing brain.

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